Characterization of the Genetic Potential, Catabolic Structure and Degradative Activities against BTEX, in Microbial Communities from Aquifers under Adaptation to Organic Contaminants

Doctoral Thesis

Howard Armando Junca Díaz

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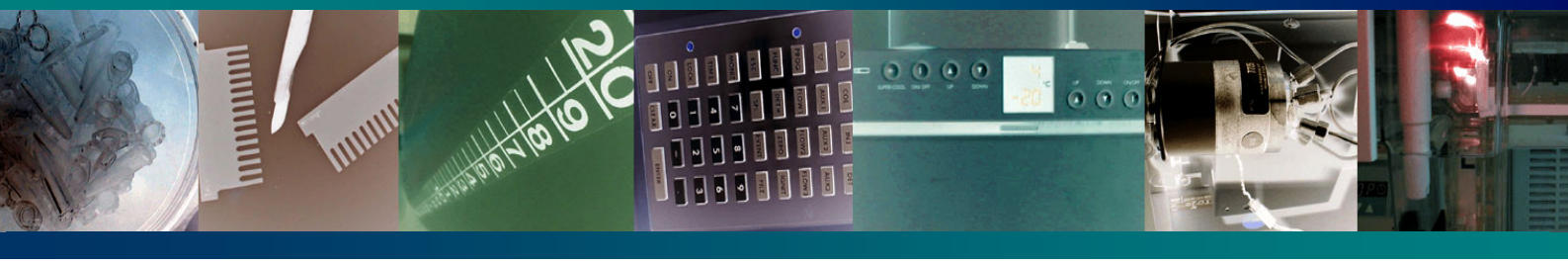

Characterization of the Genetic Potential, Catabolic Structure and Degradative Activities against BTEX in Microbial Communities from Aquifers under Adaptation to Organic Contaminants

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Summary

Developments in molecular techniques have led to rapid and reliable tools to monitor microbial community structures and dynamics under *in situ* conditions. However, even though various functional genes are localized on mobile genetic elements such that metabolic potential/activity is not necessarily reflected by the community structure, there has been a lack of emphasis on monitoring functional diversity. A more detailed picture of the catabolic gene structure and sequence diversity in environmental samples will significantly increase our knowledge of the functional potential of microbial communities. We adapted suitable techniques to follow functional gene diversity and applied those to target catechol 2,3-dioxygenases as key genes in aromatic hydrocarbon degradation.

Catabolic gene diversity in differentially BTEX contaminated environments was assessed by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP). Site specific PCR-SSCP fingerprints were obtained, showing that gene diversity experienced shifts correlated to temporal changes and levels of contamination. PCR-SSCP enabled the recovery of predominant gene polymorphs. The PCR-SSCP technique could be shown to be a powerful tool for assessing the diversity of functional genes and the identification of predominant gene polymorphs in environmental samples as a prerequisite to understand the biodegradation functioning of microbial communities.

A new method for isolating strains capable of growing on BTEX compounds was developed to diminish pre-selection or enrichment bias and to assess the function of predominant gene polymorphs. To rapidly determine phylogenetic diversity of functional genes from strain collections or environmental DNA amplifications, a restriction enzyme, theoretically producing characteristic profiles, the similarities of which reassembled the main divergent branches of C23O gene phylogeny, was used to perform an amplified functional DNA restriction analysis (AFDRA) on C23O genes of reference strains and isolates. Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the AFDRA approach. AFDRA thus provided a quick assessment of C23O diversity in a strain collection and insights of its gene phylogeny affiliation. AFDRA was also used to determine the predominant polymorphism of the C23O gene present in environmental DNA extracts and in combination with a most-probable-number-PCR approach, its abundance. This approach may be useful to differentiate functional genes also for many other gene families.

Isolates harbouring C23O genes, identical to the gene polymorph predominant in all contaminated sites analysed, showed an unexpected benzene but not toluene mineralising phenotype whereas isolates harbouring a C23O gene variant differing by a single point mutation and observed in highly polluted sites only, were capable, among some other isolates, to mineralise benzene and toluene, indicating a catabolically determined sharing of carbon sources on-site. Complete C23O encoding open reading frames were cloned, sequenced and overexpressed by using conserved regions in operon neighbouring genes. Such strategy also allows the direct access to complete genes from environmental DNA. A single amino acid substitution at position 218 had severe influence on enzyme kinetics, and the Tyr218 variant differed from the His218 variant by lower turnover number but higher affinity.

The information in this work underlines the importance to analyze catabolic gene diversity at different scales, from global views of diversity and fitness of genes in the ecosystem, to detailed understanding of sequence variations effects on catalytic activities.

CHAPTER I

General Introduction

Ecology of Biodegradation

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General Introduction

Ecology of Biodegradation

1.1 Preface

The introduction to the experimental work is structured around four topics related to biodegradation of aromatic pollutants in the environment. These topics are pollution and bioremediation, microbial ecology, molecular biology, and aerobic biodegradation pathways. The rational behind such wide coverage lies on the expectations to achieve synergic interactions between these disciplines. To be capable to understand how biodegradation proceeds and can be optimized *in-situ*, the catalysts performing degradation and their interaction need to be followed directly in the environment where the contamination occurs. To define the scope of the specific subject of this thesis in these broad action areas, suitable model ecosystems, catabolic indicator targets, techniques to analyze the information, and advantages and limitations of approaches to assess functions in microbial communities are presented and discussed.

1.2 Environmental pollution with aromatics: facts and effects

Massive amounts of petroleum hydrocarbons have been used in industrial processes for a huge kind of useful purposes, based on their ideal properties for combustion and organic synthesis. Unfortunately, such processes are never completely efficient. The extraction procedure always results in the release of a more or less considerable fraction, the conversion of the raw material to final products is not 100% efficient, and is not possible to completely use the energy released in combustions (Chow *et al.*, 2003). Therefore, in any industrial activity, to a minor or major extent, waste products are formed and often released into the environment. Virtually, there is no industrial action fully 'clean' in terms of the undesirable side production of non-profitable or toxic compounds. This environmental pollution is a serious threat for human health, ecosystem biodiversity and functioning (Jenkins, 2003). Only since the recent years, there is a global concern on the negative effects of contamination and discussions how to solve them. National and supranational regulatory bodies of many countries have got interested in the sustainable development of industrial activities and their environmental impacts, like contamination (Botello *et al.*, 1997). In Europe, the earliest and at present one of the highest industrialized regions, the massive exploitation of natural resources is considered to begin around 1820 (Halsall, 1997), but the interest on assessment or remediation of environmental impacts was politically expressed in formal agreements regulating these aspects just since 1972 (EU., 1972). Thus, there is a gap between beginning of industrial activities (and the accumulation of their associated wastes), and the application of strict regulations regarding environmental issues. While some laws have been trying to control waste management or decrease waste volumes, it is a fact that the amount of waste in Europe is increasing each year (EEA, 2002). Furthermore, the United States, the highest industrialized region in the world, are releasing through industrial activities, around 50% of the world's total gases with greenhouse effect, and until now they are not formally compromised to reduce these amounts (Chow *et al.*, 2003).

Contamination can be controlled at different levels. One one hand, after identification of critical steps in risk assessment studies, preventive measures can be applied and the release of contaminants reduced or alternative environmentally friendly technologies utilized. Often, physical containment of the contamination is chosen as method, leaving the risk behind for future generations. Alternatively,

physical, chemical or biological remediation technologies can be applied to detoxify the impacted areas. In any of these strategies, for a successful prevention, containment, or remediation, a detailed knowledge of the contaminant is essential, including sources and its fate in the environment. This involves chemical, physical and biological factors, like dispersion, toxic effects, or degradation.

One important fraction of crude oil is consisting of aromatic compounds. These compounds have been used extensively in a variety of manufacturing processes, for syntheses of plastics, resins, fibers, pesticides, pharmaceuticals, dyes, and lubricants, among others. Because of this broad range of applications, aromatic compounds are among the top chemicals by production volume used in today world's industry. Out of this group, monoaromatics are highly volatile, flammable, toxic, and carcinogenic compounds (ATSDR, 1992) commonly found as contamination linked to human activities (Baun *et al.*, 2003; Fang *et al.*, 2000; Foo, 1991; Fraile *et al.*, 2002; Kato *et al.*, 1993; Wycisk *et al.*, 2003; Zamfirescu and Grathwohl, 2001). When these compounds are released into the atmosphere, they can be transformed or degraded by physicochemical reactions (Olariu, 2001). Alternatively, the aromatic compounds can be washed out from the atmosphere, by snow or rain, resulting in their accumulation in soils and aquifers, with the consequent impact on ecosystems and drinking water. More severely, during the transportation or extraction of petroleum hydrocarbons, often a significant release occurs, and accidents or spills from the pipelines contribute to the contamination of soils, aquifers and marine ecosystems, which usually have lower concentrations of aromatic compounds (Rowland *et al.*, 2001; Yunker *et al.*, 1999). The severe effects of the oil spills on the ecosystems are well documented, and evidenced by the increased and persistent mortality of higher organisms after the Exxon Valdez accident (Lance *et al.*, 2001). According to the International Tanker Owners Pollution Federation Limited (www.itopf.com), since 1970 at least 5 million tons of oil have been spilt in marine accidents. In habitats like open oceans or underground cavities, aromatic compounds are relatively recalcitrant and the low rate of biodegradation results in their accumulation, and harmful long-lasting effects (Johnston *et al.*, 1998; Olivella *et al.*, 2002; Schluep *et al.*, 2001; Wiedemeier *et al.*, 1996).

1.3 Bioremediation and natural attenuation

Various approaches to clean contaminated environments have been proposed, including chemical, physical or biological treatments. Among them, the biological

treatment is considered as an efficient and cost saving way to achieve remediation of contaminated sites (Atlas, 1991; Chapelle, 1999; Dobbins *et al.*, 1992; MacNaughton *et al.*, 1999; Samanta *et al.*, 2002). During the previous years, natural attenuation, "naturally occurring processes in soil and groundwater that act without human intervention to reduce the mass, toxicity, mobility, volume or concentration of contaminants in those media" (Pope and Jones, 1999) has received increasing attention. Natural attenuation in soil and groundwater may include a number of processes such as biological degradation by naturally occurring microorganisms, sorption, dispersion, advection, dissolution, and volatilization. These processes are operating at practically all contaminated sites. It is generally accepted that microorganisms are the principal mediators of the natural attenuation of many pollutants. They transform or mineralize pollutants, thereby usually decreasing their masses and toxicities, in contrast to most other components of natural attenuation. However, in some cases pollutants may be transformed into more toxic products, as reported for the anaerobic transformation of trichloroethylene (TCE) (Abelson, 1990; DiStefano *et al.*, 1991; Freedman and Gossett, 1989; Maymo-Gatell *et al.*, 1995; Vogel and McCarty, 1985). The use of natural attenuation thus requires a detailed monitoring to determine how effective natural attenuation is for attaining site remediation goals. A detailed monitoring over time is costly and makes *in situ* remediation approaches unattractive. However, the long-term costs for natural attenuation may be less than for other remedial technologies. Unfortunately, tools to follow *in situ* bioremediation activities are not well established yet (Dua *et al.*, 2002).

Microorganisms and microbial communities play essential roles to maintain life on earth's biosphere, as they are performing relevant steps to complete the biogeochemical cycles, maintaining the global recycling of vital elements like carbon, oxygen, nitrogen, sulfur, phosphor, iron, or manganese, all essential for the sustainability for the different forms of life (Conrad, 1996; Conrad, 2000; Paerl and Steppe, 2003). However, the molecular processes controlling these microbial functions *in situ* are still poorly understood and theoretical frames to predict the interaction in microbial communities or their fitness are still in the early stages of development (Krüger *et al.*, 2003). The synergistic cellular programs selected and evolved for specific purposes, or the evolution of whole communities as complete functional units, are issues of particular interest, specifically when the selective conditions are events of contamination (Nikodem *et al.*, 2003; Pelz *et al.*, 1999). Microbial adaptations under these conditions can be regarded as a sort of iterative

adaptations of their metabolic interactions, shaped by exchange and sharing of metabolites, but these interactions in more complex communities are still not understood (Dejonghe *et al.*, 2001).

Considering the advances in tools to study microbial community composition and changes under environmental conditions (Torsvik and Ovreas, 2002), it is very likely to expect a logical future trend aiming to transfer these microbial ecology technologies to detect and predict biological activities very precisely, if possible at the genetic level. Only a detailed understanding of the functioning and interactions in microbial communities will allow their rational manipulation, and the overcoming of factors limiting efficient bioremediation. Moreover, such tools will allow also to reliably follow stimulation of natural (autochthonous) bacteria (Freedman and Gossett, 1989; Klecka *et al.*, 1998; Landmeyer *et al.*, 2001; Lollar *et al.*, 2001; Lu *et al.*, 1999; Macdonald, 2000; Margesin and Schinner, 2001b; Roling and van Verseveld, 2002; Salanitro *et al.*, 1997; Smets *et al.*, 2002; Solano-Serena *et al.*, 1998; Stapleton *et al.*, 2000; Sturchio *et al.*, 1998; Tuxen *et al.*, 2002; van der Meer *et al.*, 1998; Weiner and Lovley, 1998; White *et al.*, 1998; Wiedemeier *et al.*, 1996; Yager *et al.*, 1997). Using adapted native populations from the original site, the potential risks and concerns about releasing genetically modified organisms are avoided (Sayler and Ripp, 2000), and at the same time, a rapid and correct interpretation of metabolic performance will allow rational interference and decision making, to establish reliable bioremediation regimes, resulting in diminished costs. To analyze in detail interacting microbial communities and to deduce where and how adaptations important for bioremediation are occurring, it is necessary to collect the information available on microbial biodegradation pathways, to evaluate the most informative technique/indicator for the specific purpose (functional changes in communities), and to develop and validate via experimental work the suitable tools to extract such information from environmental samples (Rieger *et al.*, 2002; Watanabe, 2001).

Aromatic compounds have been discharged into the environment during the last years in increasing amounts, and they have significant impact on natural microbial communities, and thus, the global element mass fluxes. The functional diversity in nature shows that many microorganisms have the potential to degrade and recycle aromatic compounds (Watanabe *et al.*, 2002). This potential can be used for bioremediation processes mentioned above. In the next sections, the tools, the information available, and possible strategies for environmental diagnostics of aromatic degradation in the environment will be introduced.

1.4 Molecular biology tools to study the microbial ecology of biodegradation

It is known since the early years of the microbiological sciences, that by the available methods, only around 0.1 to 1% of the total viable bacterial cells present in a variety of ecosystems can be cultured

Table 1.

Habitat	Culturability (%)
Seawater	0.001–0.1
Freshwater	0.25
Mesotrophic lake	0.1–1
Unpolluted estuarine waters	0.1–3
Activated sludge	1–15
Sediments	0.25
Soil	0.3

Culturability of microorganisms in diverse ecosystems. Culturability was determined as a percentage of culturable bacteria in comparison with total cell counts (Amann *et al.*, 1995). Culturable bacteria were measured as colony forming units.

There are two alternative and complementary possibilities to expand the knowledge about this not cultured environmental bacteria: by trying to recover, applying new enrichment regimes, not-yet cultured strains and analyze their metabolism, or by adapting or developing dedicated molecular methods for the culture-independent survey of microorganisms and their functional properties. This last possibility takes advantage of the great advances in molecular biology in the last decades. Since the discovery of nucleic acids as the molecules carrying the genetic information, as evidenced by the early studies on bacterial transformation by Griffiths (1928), the determination of the chemical identity of the genetic material by Avery, McCarty and MacLeod (1944), the *in vivo* studies with viruses and sulphur radioactive isotopes by Hershey and Chase (1952), and the Watson and Crick proposal of the double helix structure of DNA (Watson and Crick, 1953), the possibilities to understand biological processes, to manipulate and modify genetic information, have reached a new age where the molecular bases of functioning and evolution of life are being deciphered. The elucidation of basic biological principles, in many cases, generated useful tools, like the discovery of splicing in viral or eukaryotic genes, which resulted in the isolation of restriction enzymes (Kelly and Smith, 1970) one of the most powerful tools in molecular biology, used e.g. to clone genes or to differentiate genes or organisms by restriction patterns (Danna and Nathans, 1971). The bacterial genetic factors conferring resistance to antibiotics and extra chromosomal genetic elements (plasmids) (Lederberg, 1952) have been extensively used as markers and genetic vectors to introduce foreign

genes in new bacterial hosts. Some other landmarks, like the separation of DNA molecules according to size by agarose-gel electrophoresis (Aaij and Borst, 1972), the logarithmic amplification of DNA fragments from minute initial amounts by Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1986), and the possibility to determine the exact nucleotide sequence of DNA fragments (Sanger *et al.*, 1973), supported a rapid evolution in several biological disciplines. The synergetic combination of different molecular biology DNA techniques provides researchers with new means to address basic questions in Biology. Nucleic acid (DNA/RNA)-based techniques have been applied to analyze genomic aspects like gene composition, promoter functions or gene expression by constructing DNA libraries and sub-libraries (Maniatis *et al.*, 1978) through fragmentation of a complete genome by enzymatic restriction or physical treatments, and further cloning, expression and sequencing of the resultant DNA fragments in vectors (plasmids, cosmids, bacteriophages). The collection of fragments representing the complete information of a genome can be analyzed for specific genes or genetic elements in a number of ways.

Some of the widely applied screening techniques are based on the hybridization properties of DNA. The annealing between complementary strands can be used to localize fragments sharing homology, but also to discriminate between single nucleotide differences in highly homologous fragments under restrictive hybridization conditions. In these cases, DNA fragments of known sequence are used as probes to analyze restricted genomic DNA (Southern, 1975), colonies or plaques containing gene libraries (Grunstein and Hogness, 1975), or total RNA (Alwine *et al.*, 1977) for the presence of a gene variant in a given genome, followed by isolation and characterization of the genes of interest. The significance of the information that has been retrieved by the application of DNA hybridization, isolating and characterizing genes, certainly represents a revolution in our understanding of biological processes in single organisms.

Recent molecular biology research has focused on the analysis of the not-yet-cultured microbial fraction, and molecular tools have been specifically developed and applied to study microbial communities at the molecular level in the environment (O'Donnell and Williams, 1991), enabling to identify changes in phylogenetic structure (predominant taxa) of microbial communities under changing conditions. The pioneering work of Woese introduced the classification of bacteria based on 16S rRNA sequences conservation (Woese *et al.*, 1975). This new path of molecular taxonomy resulted, among others, in the proposal of a new

Kingdom, the *Archaea* (Woese and Fox, 1977). The isolation and identification of 16S rDNA sequences directly from environmental samples without culturing, by extracting total DNA from environmental samples, PCR amplification using primers annealing to the known conserved regions of the 16S rDNA gene, generation of libraries of these PCR fragments (16S rDNA PCR clone libraries) for random sequencing of a numerous amount of inserts coupled to phylogenetic analyses, revolutionized and expanded our knowledge on microbial diversity in the environment (Giovannoni *et al.*, 1990; Ward *et al.*, 1990). These and subsequent studies applying these techniques, helped to realize the extreme diversity of the not yet cultured bacterial fraction present in nature. More than 60.000 different 16S rDNA sequences have been reported thus far, and a gram of soil is estimated to contain 10^{-5} to 10^{-9} bacterial cells, comprising representatives of around thousand different species (Rosselló-Mora and Amann, 2001). This supports, that for many ecosystems, the non cultured fraction has a taxonomic diversity exceeding by far the current global diversity inferred from cultured isolates (Amann *et al.*, 1995). The advances in nucleic acids extraction from environmental samples, automated sequencing, miniaturized hybridization devices and detection systems, which combine the advantages of these and other developments in the Computational Biology, Nanotechnology, Bioinformatics, Electron Microscopy and Digital Image Processing fields, permitted the development of new applications for Microbial Ecology studies. For example, Fluorescent *in situ* hybridization (FISH) (Cheung *et al.*, 1977), helps to characterize the spatial distribution of specific taxonomic or functional groups using discriminative oligonucleotides probes, and is particularly useful in studies of microbial biofilms (Klausen *et al.*, 2003; Martiny *et al.*, 2003). DNA microarrays (Lucchini *et al.*, 2001) are able to evaluate, via automated hybridization screenings based on oligonucleotide probes, the relative amounts or variations in hundreds of different genes. This technique, first intended for transcriptome analysis (e.g. to detect transcripts expressed or silenced under certain physiological states of a culture), is now under development for detection of changes in microbial community composition using oligonucleotides of variable 16S rDNA gene regions to discern specific taxonomic groups (phylogenetic chip) (Loy *et al.*, 2002) or even to detect functional genes in microbial community DNA (Denef *et al.*, 2003; Valinsky *et al.*, 2002). Another technique applied in microbial ecology is flow cytometry (Van Dilla *et al.*, 1969), which combines developments of capillary cell sorting and immunodetection (Valet, 2003), allowing to separate and quantify populations of cells of different kind of shapes or complexities.

Applications range from biomedical research, e.g. detection and quantification of cell subpopulations in higher organisms like cancer cells or specific cells of the immune response (Kussick and Wood, 2003), to applications in limnology to detect variations in phytoplankton populations (Andreatta *et al.*, 2001). This technique is expected to provide an alternative approach to analyze the non cultivable organisms on the genomic level, by sorting and concentrating specific populations in amounts high enough, to extract nucleic acids and construct genome libraries (Eilers *et al.*, 2000), which would help to identify the genetic basis of the functions microorganisms have in the environment, an information hardly accessible by classical culture techniques.

However, results of culture independent studies need to be integrated with information obtained from isolates. Thus, in microbial ecology or biodegradation studies, extensive strain collections used to be generated. Moreover, by varying enrichment regimes and developing new culture conditions, an increased number of microorganisms previously thought to be uncultivable, could in fact be isolated and cultivated (Kaeberlein *et al.*, 2002; Zengler *et al.*, 2002).

Techniques to analyze genomes, like detailed hybridization analyses, construction and screening of gene libraries, or time consuming sequencing are not practicable strategies if the aim is to compare a large numbers of isolated strains at genus, species, or strain level.

Molecular fingerprint methods can be very useful to discern distinct representatives in a strain collection without previous knowledge of genome sequence information of the organisms analyzed. Some of the most widely used methods are Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP). AFLP (Yunis *et al.*, 1991) consists of the selective amplification of restriction fragments from a genome or cDNA, ligated with small complementary dsDNA adapters. These fragments are then amplified with primers annealing to the restriction site sequences plus the defined additional nucleotides, and the different length fragments can be resolved on gels. These genomic fingerprints are useful for biodiversity studies or genetic distance analyses in strain collections. Another similar approach takes advantage of arbitrary priming in unknown genomes to generate strain-specific amplification patterns. This strategy is exploited in RAPD (Williams *et al.*, 1990), a PCR technique that uses a short primer (between 8-14 nucleotides) of a randomly selected sequence, and low annealing temperatures, to produce a characteristic pattern of amplification products of different sizes from a

specific complex DNA template (e.g. genomic DNA or environmental DNA). RAPD proved to be a very useful tool to discriminate between closely related organisms from which no genomic information is available. RFLP (Hillis *et al.*, 1996) is one of the first reported methods for molecular fingerprint generation, and it can be used to analyze relatively short DNA fragments as those generated by PCR, but also very large DNA molecules and even complete genomes. Upon restriction with a single or a mixture of restriction enzymes, characteristic fragment profiles are produced. These fragments are resolved by gel electrophoresis and detected, depending of the degree of selection to achieve, by staining (for relatively simple mixtures, e.g. in phylogenetic studies to analyze diversity in PCR products of gene families), or by hybridization with a defined probe (when many restriction fragments of the same size, but different in sequence are present, e.g. in restrictions of complete genomes). As the profiles produced directly reflect differences in sequence, similarities of profiles can be interpreted in many cases as indicator for evolutionary relationship. These fingerprinting techniques can be optimized to give additional information, in terms of quantity, like in Quantitative (Q)-PCR (Ferre, 1992; Larzul *et al.*, 1988) or of gene expression by Reverse Transcription (RT)-PCR (Baltimore, 1970; Lee *et al.*, 1989; Temin and Mizutani, 1970).

A fingerprinting technique dedicated to compare gene expression profiles is Differential Display (DD)-PCR (Liang and Pardee, 1992; Welsh *et al.*, 1992). This technique takes advantage of arbitrary priming, but restricts the amplification to randomly reverse transcribed RNA's. It allows the characterization of genes active at the transcriptional level under specific environmental conditions by comparing expression of genes of organisms under different physiological states or treatments. This technique is also suitable to define differentially expressed genes at the complexity level of microbial communities.

Various fingerprint techniques have been developed to rapidly detect differences in gene sequence (understood as mutations or polymorphisms), and to follow changes in the diversity of specific genes in single genomes and microbial communities. Specifically the diversity in 16S rDNA amplifications from environmental samples received attention, as such diversity directly gives an indication of the taxonomic composition of a given community. To avoid the time consuming generation and random sequencing of numerous PCR clone libraries, microbial ecologists have been actively working on developing and refining fingerprint techniques. However, polymorphisms are not only very informative from an evolutionary standpoint as they reflect the phylogenetic relationships and

common ancestors of the sequences in given gene families. Perhaps one of the most interesting aspects to study gene polymorphisms is that small sequence changes can have significant impact on the functional properties (activities) of the encoded enzymes and, thus, specific variants will be selected under environmental conditions based on the functional fitness of the evolved protein. Thus, gene polymorphisms could be meaningful also at the biochemical level, specifically if information on protein sequence determinants essential to maintain or modify a defined function is available.

Denaturing or Temperature Gradient Gel Electrophoresis, DGGE or TGGE (Muyzer *et al.*, 1993; Riesner *et al.*, 1989) belong to the most relevant techniques. These methods rely on the difference in melting behavior of two complementary DNA strands upon heat (TGGE) or chemical denaturants (DGGE) application, which depend on the G+C content and is thus sequence dependent. If mixtures of homologous DNA fragments are subjected to electrophoresis on gels with gradients of the denaturing factor (gradually increasing temperature or denaturant), specific mobilities depending on the sequence (G+C content) will be attained. These techniques have been applied for comparison of single genes, for screening of single nucleotide polymorphisms (SNP), or to resolve complex amplicon mixtures. They were used predominantly to analyze the diversity of 16S rDNA fragments amplified from environmental samples. More recently, they have been used also to analyze diversity in environmental functional genes (Felske *et al.*, 2003; Henckel *et al.*, 1999; Rosado *et al.*, 1998; Watanabe *et al.*, 1998).

Another powerful fingerprint technique is Single Strand Conformation Polymorphism (SSCP) (Orita *et al.*, 1989), originally intended to screen for single point mutations between two genes. Under non-denaturant conditions, single stranded DNA acquires a secondary conformation, which depends on the sequence. Thus, two DNA fragments of the same size but of different sequence will fold differently, and these sequence dependent conformations can be resolved in non-denaturant polyacrylamide gels. Actually, this technique is one of the most effective and widely uses methods to identify SNPs between gene fragment amplifications from two specimens. SSCP was later optimized to analyze only one of the complementary single strands (Schwieger and Tebbe, 1998), by preferentially degrading with lambda exonuclease the one strand generated with a phosphorylated primer (Fig. 1). This development aims to avoid heteroduplex formations, or overlapping of forward-reverse strands from different amplicons during separation, allowing the separation of mixtures of fragments of identical

size but different in sequence. The application of this modified technique was focused on studies of taxonomic shifts in microbial communities by targeting 16S rDNA genes (Peters *et al.*, 2000; Schmalenberger *et al.*, 2001; Schmalenberger and Tebbe, 2003; Schwieger and Tebbe, 2000), however, a potential application to assess diversity of functional genes was foreseen (Stach and Burns, 2002).

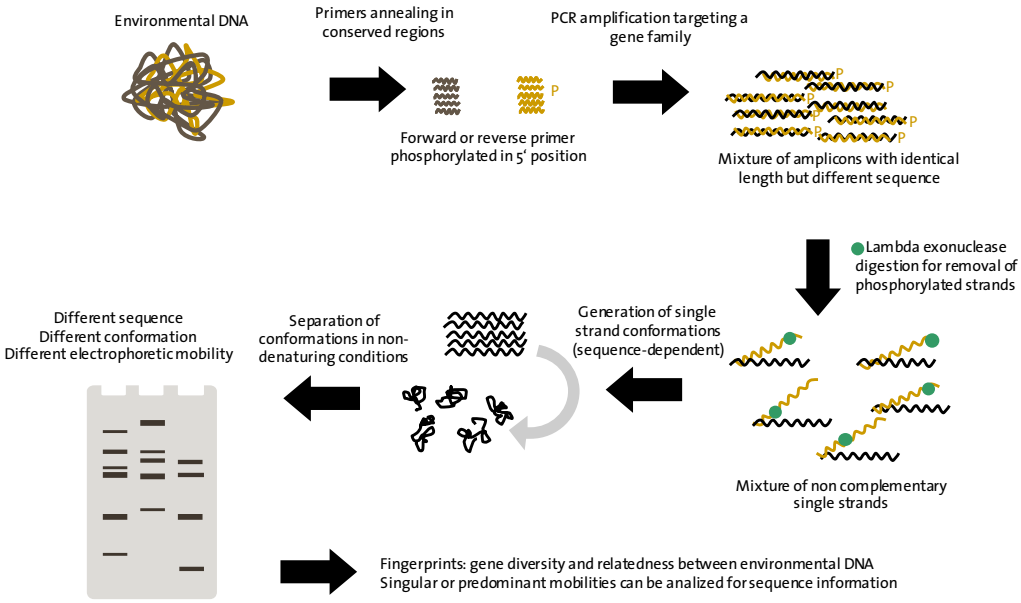

Fig. 1. Schematic representation of the modified PCR-SSCP technique (Schwieger and Tebbe, 1998) to analyze complex amplicons

All the fingerprint methods mentioned above allow to recover genetic information from the profiles generated, by means of excising bands of interest, and reamplifiying and sequencing of the respective PCR fragments, the information of predominant or emerging bands associated with a culture or environmental condition can be determined. Another technique to analyze complex amplicon mixtures is Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu *et al.*, 1997). In contrast to PCR-RFLP, two labelled primers are used, such that terminal restriction fragments become labelled and can be detected by an automatic sequencer. T-RFLP analysis is a highly reproducible and robust technique that yields high-quality fingerprints consisting of fragments of precise sizes. Like other fingerprinting methods, it has been mainly used to analyze microbial community composition and changes by analyzing diversity in 16S rDNA genes. However, patterns produced from amplified 16S rDNA are simplified representations of the community, and different species may produce identical length terminal fragments with a given restriction enzyme, such that a careful selection of restriction enzymes giving meaningful taxonomic information, is necessary (Kent *et al.*, 2003; Osborn *et al.*, 2000). Few attempts are reported to

transfer this technology to functional genes (e.g. Braker *et al.*, 2001; Tan *et al.*, 2003), mainly because of the limited range of suitable target gene families, necessitating on one hand a detailed survey on sequence information and diversity, and on the other the presence of phylogenetically informative restriction sites (Lueders and Friedrich, 2003).

As can be noted, various approaches focus currently on an understanding of functional properties in microbial communities and on the elucidation of community members important for such functions. Recently, two methods based on the use of labeled isotopes have been developed to identify community members carrying out important metabolic functions. One of these methods use radioactive labeled substrates, relying on the fact that organisms mineralizing those substrates incorporate radioactive label in their cell material. FISH technique using phylogenetic probes was then used to characterize, on the phylogenetic level, active organisms (Gray et al., 2000; Lee et al., 1999; Ouverney and Fuhrmann, 1999). Stable isotope probing take use of non-radioactive heavy isotopes such as ^{13}C. Incorporation into phylogenetically important biomarkers, specifically lipids (Abraham *et al.*, 1998; Boschker *et al.*, 1998; Roslev *et al.*, 1998) was used to identify community members involved in the metabolism of the ^{13}C labeled substrates. Recently, this method was adapted to analyze nucleic acid as phylogenetically most informative molecules. It could be shown that ^{13}C-DNA, produced during the growth of metabolically distinct microbial groups on a ^{13}C-enriched carbon source, can be resolved from ^{12}C- DNA by density-gradient centrifugation (Radajewski *et al.*, 2000). Later on, stable-isotope-labeled RNA was used to identify, after amplification and sequencing of PCR clone libraries or via fingerprint generation, metabolically active community members (Manefield *et al.*, 2002; Radajewski *et al.*, 2000).

Molecular ecology has, by using the broad set of methods mentioned above, gained a detailed understanding on microbial community structure and diversity in various environmental systems. Studies characterizing functional gene diversity are part of a research tendency combining elements, principles and techniques of microbial ecology and biodegradation disciplines. It is commonly encountered that some of the specific characters playing "functional roles" (Maron *et al.*, 2003), are selected or present independently of the rate of evolution of the species diversity and taxonomy, as could be the case for phenotypic features carried on plasmids (Lederberg, 1952) or on other mobile genetic elements (Davison, 1999). Specifically in the case of aerobic degradation of aromatics, the respective genetic

determinants are predominantly localized on highly mobile genetic elements (Reineke and Knackmuss, 1979; Tan, 1999; van der Meer and Sentchilo, 2003). Even though molecular technologies developed to follow changes in the composition of the 16s rDNA gene pool can be transferred to target functional characters present in environmental isolates or in environmental DNA/cDNA PCR amplifications, practical developments of new tools, or adaptations of the existing ones, are needed and particularly useful in fields aiming to detect genes coding for enzymes important for degradation of environmental pollutants (Widada *et al.*, 2002). Considering the different scales of complexity to study gene polymorphisms, ranging from gene diversity structure and changes in the community, to critical mutations affecting the performance of the encoded proteins, the possibilities available to follow functional characters are very diverse. In figure 2, biological components that can be used as microbiological indicators through application of the tools available are shown.

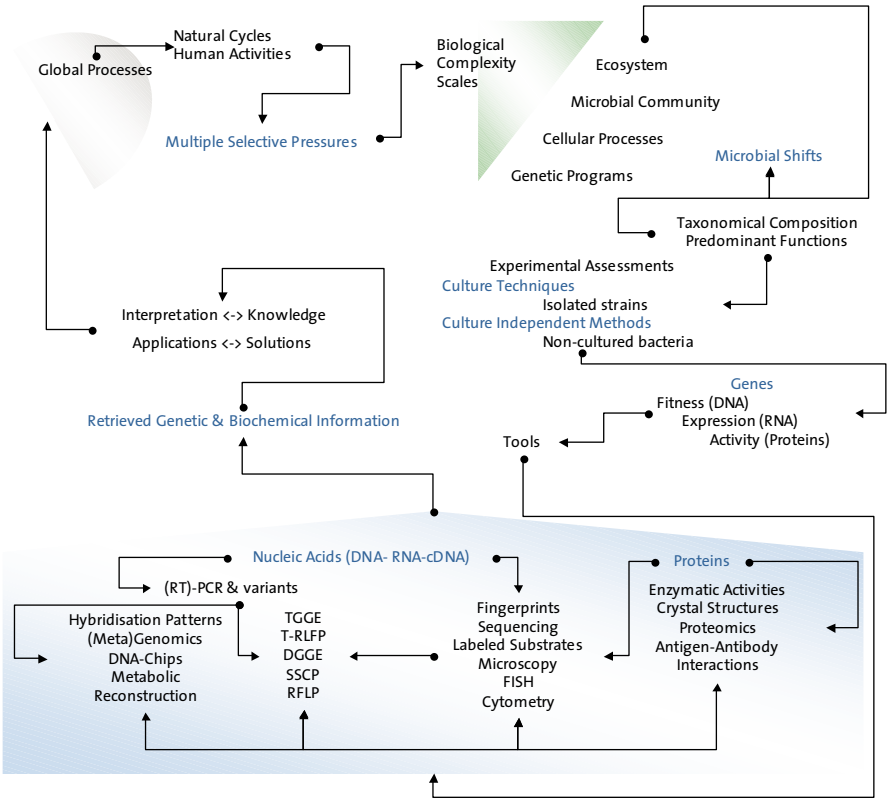

Fig. 2. Complexity scales, microbial processes and molecular approaches to study microorganisms in the environment. Several biological components can be used as microbiological indicators through application of the tools available. The bacterial community/cell is interacting with the natural (ecosystem) or artificial (culture condition) environment. The results of these interactions, the bacterial responses and the molecular indicators will vary depending of the cellular programs, lying in diverse responding genetic backgrounds.

A careful assessment of the most informative approach to extract representative data from the environment under study should be done. This should start with the

definition of the relevant microbiological questions to be answered (what are the major functional changes during adaptation of microbial communities to monoaromatic contamination), a survey on the genetic, biochemical and physiological information available (biodegradation pathways and catabolic potential shifts in the microbial community), and the range of tools (molecular fingerprints) that can describe various indicators (catabolic genes). Thus, functional indicators can be defined considering how accurate they are reflecting the catabolic potential of a environmental microbial community. This discrimination could be based on the biochemical, genetic, and environmental information about the relevance of specific functions in aromatic biodegradation pathways.

1.5 Deciphering biodegradation pathways of aromatic compounds

Prokaryotic organisms are very diverse and versatile, being able to adapt their metabolisms to recalcitrant and toxic substrates or to harsh environmental conditions (Batten and Scow, 2003; Bonch-Osmolovskaya *et al.*, 2003; Gonzalez-Toril *et al.*, 2003; Ma *et al.*, 2003; Margesin and Schinner, 2001a; Rosselló-Mora *et al.*, 2003).

Many microorganisms have evolved specialized biodegradative pathways to use aromatic compounds as a sole carbon and energy source (Harwood *et al.*, 1998; Harwood and Parales, 1996; Smets and Pritchard, 2003; Watanabe, 2001; Williams and Sayers, 1994). The biodegradation of aromatics by microorganisms can proceed under aerobic but also under anaerobic conditions. The first observations on biodegradation of aromatics were made in aerobic organisms (Stanier, 1947), and it was believed for decades, that the degradation of aromatics necessitates oxygen and that anaerobic metabolism was absent. However, even though the majority of studies dealt with aerobic degradation, the significant contribution of anaerobic degradation on the depletion of aromatics under environmental conditions came more and more into the focus (Gibson and Harwood, 2002). Specifically in the recent years, information on organisms and pathways responsible for degradation of aromatics such as toluene or naphthalene under anaerobic conditions is accumulating (Boll *et al.*, 2002), and it became clear, that aromatic degradation occurs under nitrate-, iron-, or sulfate-reducing conditions and even under methanogenic conditions. However, whereas detailed biochemical and genetic information is available on aerobic degradation of aromatic pollutants, our knowledge on degradation of aromatic pollutants under anaerobic conditions is still rather fragmentary. Significant advances have been made in recent years,

and genetic determinants for various key steps in anaerobic aromatic degradation have been elucidated (Fig. 3).

Under anaerobic conditions, aromatic hydrocarbons are initially attacked by novel, formerly unknown reactions and oxidized further to benzoyl-CoA, a common intermediate in anaerobic catabolism of many aromatic compounds (Boll and Fuchs, 1995). Toluene degradation is initiated by an unusual addition reaction of the toluene methyl group to the double bond of fumarate to form benzylsuccinate (Beller and Spormann, 1997a).

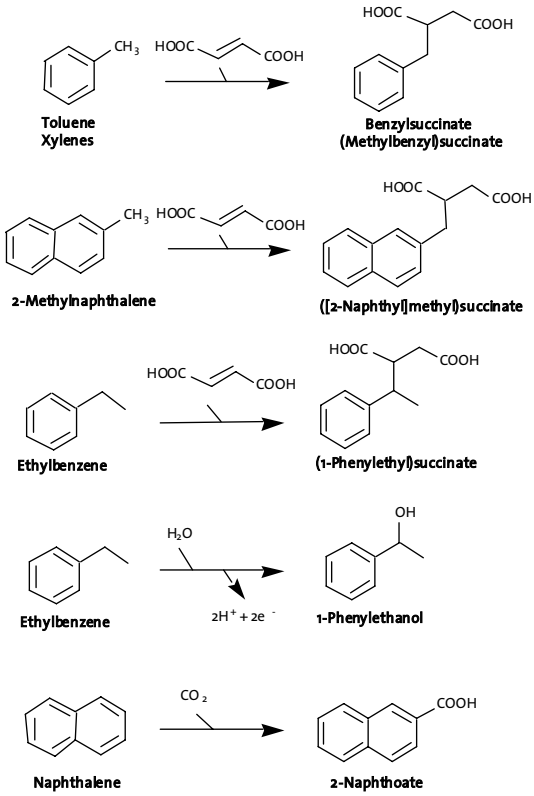

Fig. 3. Key reactions initializing the anaerobic degradation of aromatics.
The degradation of toluene, xylenes and methylnaphthalene is initiated by the addition of a methyl group to the double bond of fumarate (Beller and Spormann, 1997a; Boll *et al.*, 2002). Similarly, ethylbenzene can be activated, with activation occurring at a secondary carbon atom (Kniemeyer and Heider, 2001). Alternatively ethylbenzene can be dehydrogenated by ethylbenzene dehydrogenase to give 1-phenylethanol (Johnson *et al.*, 2001; Rabus *et al.*, 2002). Naphthalene is carboxylated by thus far unknown enzyme systems (Annweiler *et al.*, 2002; Phelps *et al.*, 2002).

The enzyme catalyzing this first step (Fig. 3), benzylsuccinate synthase, has been characterized at both the biochemical and molecular level as a unique type of glycyl-radical enzyme (Leuthner *et al.*, 1998). The further catabolism of benzylsuccinate to benzoyl-CoA and succinyl-CoA occurs via reactions of a modified beta-oxidation pathway (Leuthner and Heider, 2000). Recent studies have shown that analogous fumarate addition reactions are involved in the anaerobic activation of a variety of other compounds, including *m*-xylene, *m*- and *p*-cresol, certain *n*-alkanes, and 2-methylnaphthalene (Annweiler *et al.*, 2000; Krieger *et al.*, 1999; Kropp *et al.*, 2000; Müller *et al.*, 2001; Müller *et al.*, 1999; Rabus *et al.*, 2001). Even though benzylsuccinate synthase has been initially purified and genes identified from toluene-degrading, denitrifying (Beller and

Spormann, 1999; Leuthner and Heider, 1998) *Thauera* and *Azoarcus* strains (Achong *et al.*, 2001; Coschigano *et al.*, 1994; Leuthner *et al.*, 1998) benzylsuccinate synthase activity is distributed across a wide range of phylogenetically and physiologically diverse bacteria, including sulfate-reducing (Beller and Spormann, 1997b; Rueter *et al.*, 1994), anoxygenic phototrophic (Zengler *et al.*, 1999) and iron reducing (Kane *et al.*, 2002) strains, as well as a highly enriched methanogenic consortium (Beller and Edwards, 2000). In contrast to the information on degradation of toluene and xylenes, information on anaerobic degradation of naphthalene (Annweiler *et al.*, 2002; Phelps *et al.*, 2002) and benzene is scarce (Coates *et al.*, 2001; Coates *et al.*, 2002). Moreover, information on conservation of genes or gene sequences is not comprehensive, and specifically, information on substrate specificities of catabolic key enzymes is practically absent (Verfurth *et al.*, 2004). This limits the application of molecular environmental surveys on diversity of crucial genes. The mechanisms of anaerobic biodegradation need to be characterized and exploited to their finer details, like it has been done in the studies for aerobic degradation pathways, where more detailed biochemical and genetic information has been obtained thus far.

However, despite the broad range of information on the biochemistry and genetics of aromatic degradation, to precisely built predictive models is still a tough issue (Reardon *et al.*, 2002; Vinas *et al.*, 2002). To have such information represents a major advantage for molecular surveys on diversity and changes of catabolic genes, where key reactions of the degradation pathways for a particular contaminant shall be recognized, and genetic information of the genes coding for this steps should be available, allowing the selection of suitable catabolic indicators and their accurate detection by molecular methods to follow changes in such metabolic potential. The general reactions occurring in the aerobic degradation pathways will be described in the following section.

1.6 Aerobic biodegradation pathways of aromatics

The capability to aerobically degrade aromatics is widely distributed among different bacterial taxa. Metabolic pathways and encoding genes have been reported and characterized in many bacterial strains, predominantly belonging to Gram negative bacteria of the genera, *Pseudomonas* (Stanier, 1947), *Burkholderia* (Johnson and Olsen, 1997), *Ralstonia* (Johnson and Stanier, 1971), *Sphingomonas* (Schmidt *et al.*, 1992), *Comamonas* (Locher *et al.*, 1989), *Alcaligenes*, and to Gram positive bacteria of the genera *Rhodococcus* (Kaminski *et al.*, 1983), and

Mycobacterium (Guerin and Jones, 1988). A simplified overall comparison of the aerobic pathways for biodegradation of aromatic compounds, identified some common metabolites and steps shared among them (Fig. 4 and Fig.5).

Under aerobic conditions, aromatic compounds are prepared for ring-cleavage by the introduction of hydroxyl functions, usually in *ortho*-position to one another. Thus, the degradation of a broad range of aromatics proceeds via a few central intermediates such as catechol, gentisate, protocatechuate and hydroxyhydroquinone (Smith, 1990) (Fig. 4).

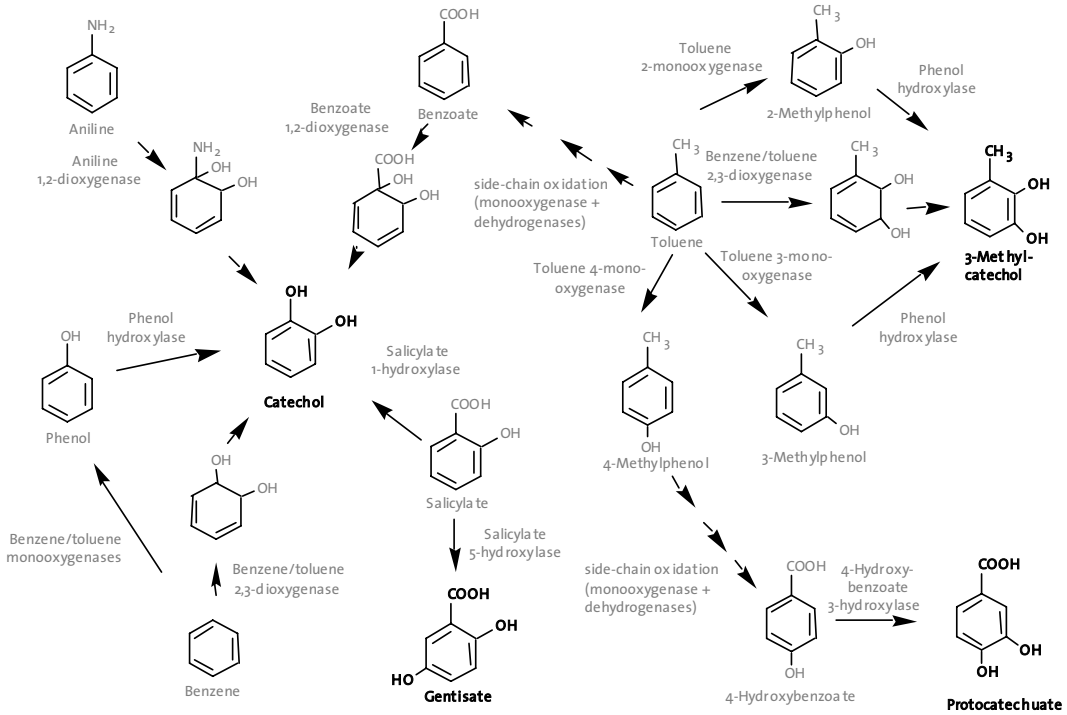

Fig. 4. Convergent metabolites in aerobic aromatic biodegradation pathways. Peripheral funneling pathways leading to the formation of central diphenolic intermediates catechol, methylcatechol, protocatechuate or gentisate (intermediates in bold letters).

Toluene has been extensively used as a model compound for aromatic degradation. Very diverse mechanisms for the initial activation of the ring has been found, performed by different enzyme systems. Thus far, five different routes for aerobic toluene activation have been described including 2,3-dioxygenation to give *cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene (*cis*-toluene dihydrodiol) (Gibson *et al.*, 1970b), monooxygenations of the aromatic ring to give *o*-cresol (Shields *et al.*, 1989), m-cresol (Olsen *et al.*, 1994), or *p*-cresol (Whited and Gibson, 1991b), respectively, or monooxygenation of the methyl function to give benzylalcohol (Worsey and Williams, 1975). These intermediates are further transformed to 3-methylcatechol (from *cis*-toluene-dihydrodiol, *o*- and *m*-cresol), catechol (from

benzylalcohol) or protocatechuate (from *p*-cresol) which are subject to ring-cleavage and then metabolized to Krebs cycle intermediates (Fig. 5).

The genes encoding the enzymes for aromatic degradation are commonly found in operons and often located on plasmids. The archetypal pathway of toluene degradation, the TOL pathway, is encoded on plasmid pWW0 of *P. putida* mt-2 (Greated *et al.*, 2002; Williams and Murray, 1974). This plasmid of 116 kb contains, between others, catabolic and regulatory genes, comprised in 39 kb of sequence lenght, conferring the ability to grow on toluene, *m*- and *p*-xylene and 1,2,4-trimethylbenzene as sole carbon sources. These *xyl* genes, are clustered in two

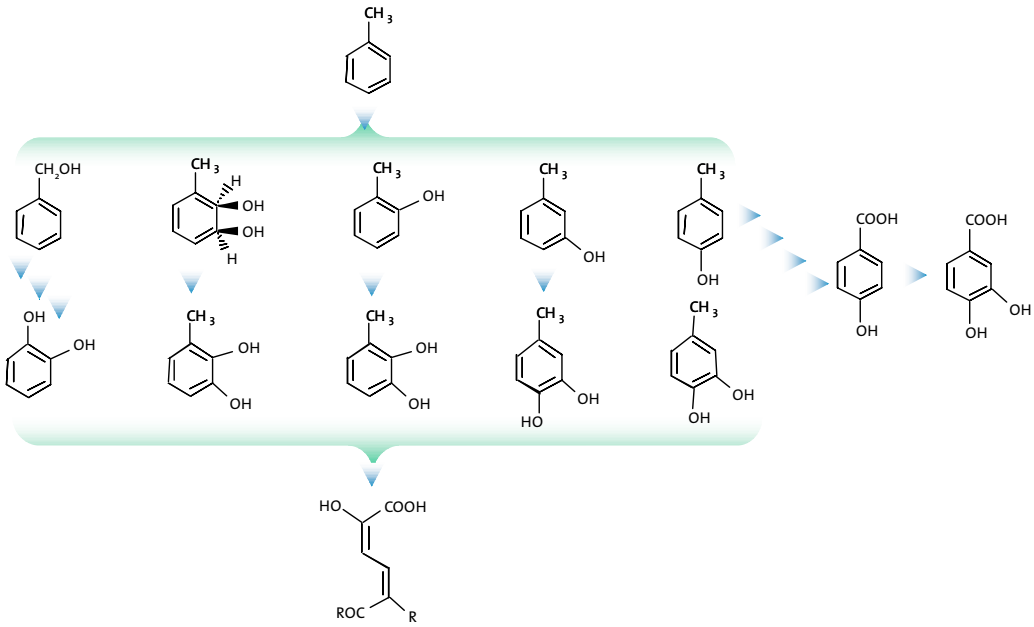

Fig. 5. Aerobic pathways for toluene aromatic biodegradation. Toluene degradation can be initiated by monooxygenation of the methyl function with benzoate (Worsey and Williams, 1975) and catechol as intermediates, by 2,3-dioxygenation with *cis*-toluene dihydrodiol (Gibson *et al.*, 1970b) and 3-methylcatechol as intermediates, or by monooxygenations of the aromatic ring to give *o*-cresol (Shields *et al.*, 1989), m-cresol (Olsen *et al.*, 1994), or *p*-cresol (Whited and Gibson, 1991b), respectively. Extradiol ring-cleavage of intermediate catechols is a key reaction common to most aerobic toluene degradative pathways.

operons, with the *xylUWCMABN* operon encoding enzymes for sequential oxidation of the substrates to the respective benzoates (upper pathway) (Harayama *et al.*, 1986; Harayama *et al.*, 1989) and the *xylXYZLTEGFJQKIH* operon encoding enzymes for the oxidation and decarboxylation of benzoates to catechols, the *meta*-fission of catechols and the further processing of the metabolites to Krebs cycle intermediates (lower pathway) (Harayama and Rekik, 1990) (Fig. 6).

Toluene degradation initiated by dioxygenation has best been studied in *P. putida* F1 (Gibson *et al.*, 1968a; Gibson *et al.*, 1968b). The enzymes of this pathway are encoded in the TOD operon (*todFC1C2BADEGIH)* and comprise the toluene 2,3-

dioxygenase, toluene dihydrodiol dehydrogenase, as well as catechol 2,3-dioxygenase and enzymes for further metabolism of the ring-cleavage product (Lau *et al.*, 1994; Zylstra and Gibson, 1989) (Fig. 7).

Activation of toluene by monooxygenation has been described in *o*-, *m*-, and *p*-position. Strains capable to mineralize toluene by such pathways usually harbour a monooxygenase system to activate toluene as well as a second monooxygenase capable to transform the formed phenolic metabolites into catechols and a *meta*-cleavage pathway. Strains like *P. mendocina* KR1, however, degrading toluene via 4-methylphenol and protocatechuate (Wright and Olsen 1994), do not necessitate the presence of a phenol hydroxylase activity. One of the best studied systems for

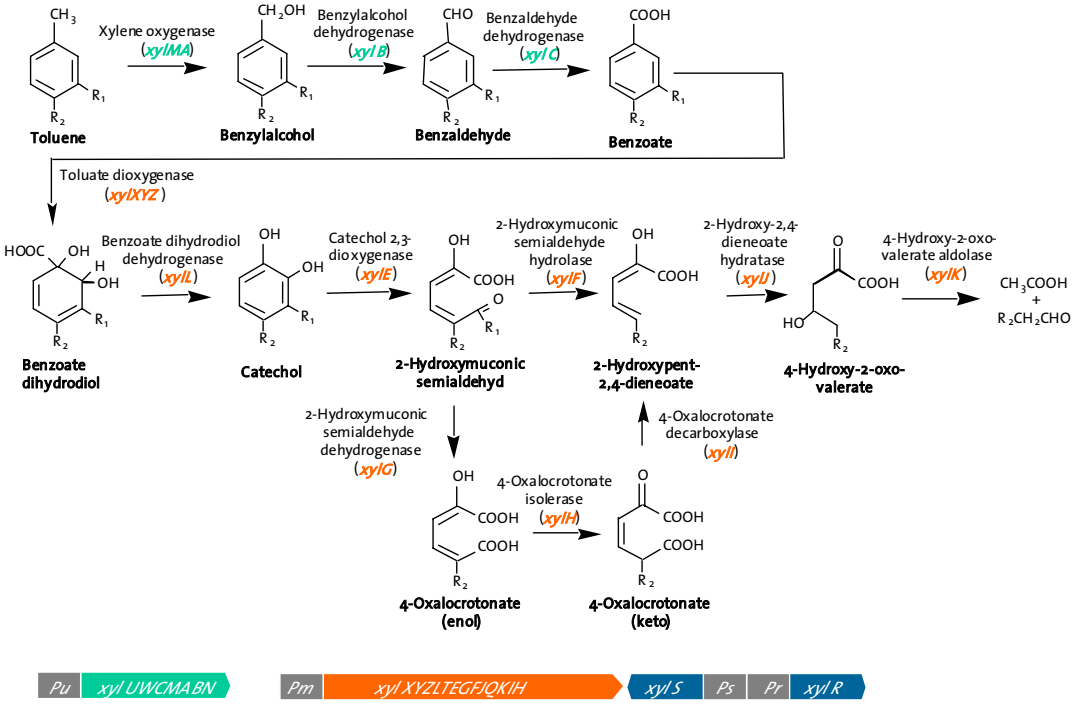

Fig. 6. Archetypal TOL pathway for toluene biodegradation. Pathway for the degradation of toluene (R_1=R_2=H), *m*-xylene (R_1=CH_3, R_2=H), *p*-xylene (R_1=H; R_2=CH_3) and 1,2,4-trimethylbenzene (R_1=R_2=) as encoded by TOL plasmids. Names of intermediates are those formed during toluene metabolism. The genetic organization is indicated at the bottom, including the Pu and Ps promoters, the regulatory proteins XylR and XylS as well as their promoters Pr and Ps (Assinder and Williams, 1990)

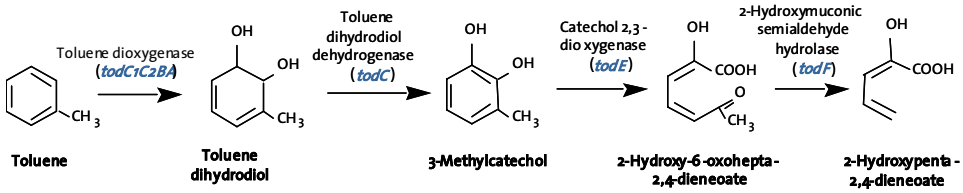

Fig. 7. Pathway for the degradation of toluene as encoded by TOD pathway of *P. putida* F1.
For gene organization see Fig. 8.

the degradation of methylsubstituted phenols is *Pseudomonas sp.* CF600. Its DMP operon (Shingler *et al.*, 1989; Shingler *et al.*, 1992), consists of 15 genes, *dmpKLMNOPQBCDEFGHI*, the first six of which encode the phenol hydroxylase subunits (Fig. 8). The following *dmpQBCDEFGH* genes encode enzymes for the

meta-cleavage of catechol. The order of the genes is identical to that observed for the *xyl* lower pathway operon. Such an organization of a multicomponent phenol hydroxylase gene cluster linked to *meta*-cleavage pathway genes is frequently observed (Barbieri *et al.*, 2001; Nordlund *et al.*, 1993). In *Pseudomonas stutzeri* OX1 it is assumed that such a multicomponent phenol hydroxylase/*meta*-cleavage pathway operon together with the toluene/o-xylene monooxygenase encoding operon form a complete metabolic route for toluene and o-xylene (Barbieri *et al.*, 2001) (Fig. 8). A slightly different gene organization is observed in *R. pickettii* PK01 (Kukor and Olsen, 1991).

In the next sections, the genetic and biochemical information on key genes and enzymes involved in the degradation of benzene, toluene and xylenes will be briefly introduced.

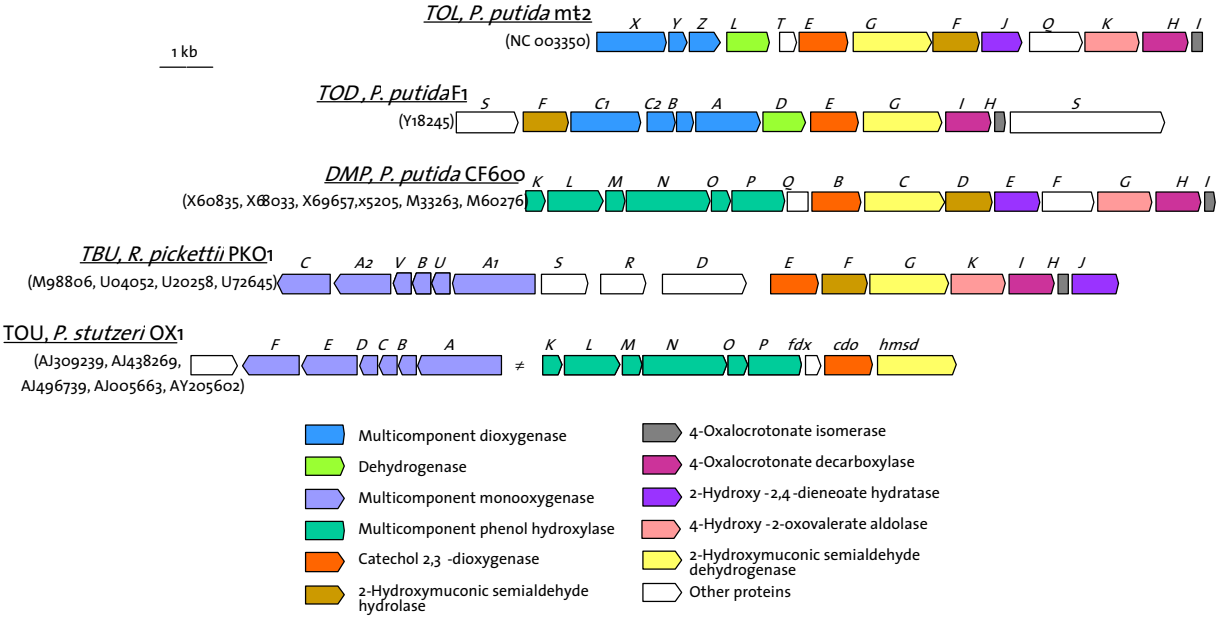

Fig. 8. Arrangement and composition of catabolic gene operons. Organization of gene clusters encoding the catechol *meta*-cleavage pathway. The lower TOL pathway operon of *P. putida* mt-2, the TOD operon of P. putida F1, the DMP operon of *Pseudomonas* sp. CF600, the *tbu* operon of *R. pickettii* PK01 and the *tou* operon of *P. stutzeri* OX1 are shown.

1.7 Oxygenases in the degradation of aromatics

The activation of the aromatic ring, through oxygenation, is a first common step to aerobic degradation pathways. As mentioned before, the oxygenation can be achieved by two kinds of reactions: dioxygenation or monooxygenation. Iron (or in some cases other metals) is playing a crucial role in the electron transfer and oxidation process (Kovacs, 2003).

1.7.1 Ring hydroxylating dioxygenases

Bacterial ring hydroxylating dioxygenases produce *cis*-dihydrodiols (eukaryotes *trans*-dihydrodiols) (Gibson *et al.*, 1970a), which are converted by NAD-dependent *cis*-dihydrodiol dehydrogenases (Patel and Ornston, 1976) into aromatic products. Ring-hydroxylating dioxygenases require oxygen, ferrous iron (Fe^{2+}) and reduced pyridine nucleotides for catalysis (Gibson and Parales, 2000). They are soluble, multicomponent enzyme systems comprising two or three separate proteins (Fig. 9). In principle, these enzymes consist of an electron transport chain that channels the electrons from NAD(P)H to the catalytic terminal oxygenase component where substrate transformation takes place. The electron transport chain is composed of either an iron-sulphur flavoprotein reductase or two separate proteins, a flavoprotein reductase and an iron-sulphur ferredoxin. The catalytic terminal oxygenase component is also an iron-sulphur protein, which is arranged as a homo- or heteromer. The heteromeric oxygenase components consist of a large α-subunit of about 50 kDa (equivalent to the homomeric dioxygenase subunit) and a small β-subunit of about 20 kDa in size. The α-subunit contains an active-site non-heme mononuclear Fe^{2+} center and the substrate binding site. In addition, all terminal α-subunit monomers contain one Rieske-type [2Fe-2S] cluster (Mason and Cammack, 1992) (Fig. 9).

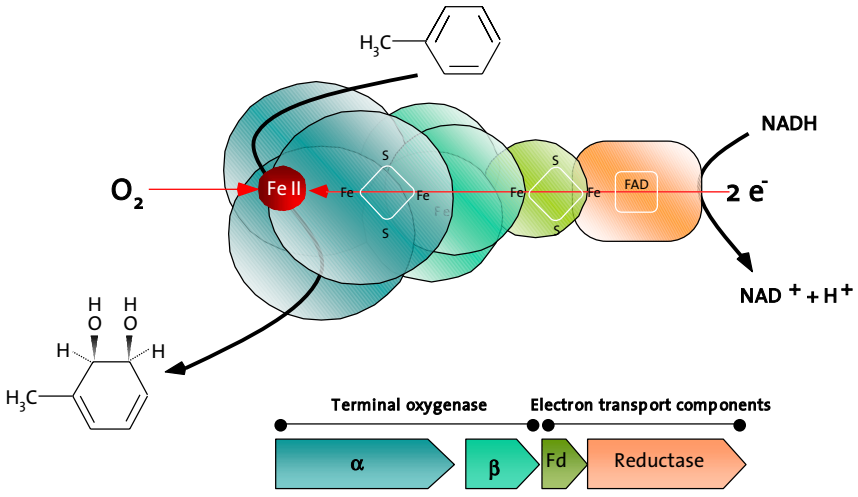

Fig. 9. Structural organization of toluene 2,3-dioxygenase enzymes. Biochemical organization of toluene 2,3-dioxygenase.Two electrons are transferred from NADH *via* the electron transfer chain, consisting of reductase containing a flavin redox center, and ferredoxin (Fd) containing a Rieske-type [2Fe-2S] iron-sulfur redox center, to the terminal oxygenase component, comprising the small β-subunit (β) and the large α-subunit (α). The incorporation of the two atoms of the dioxygen molecule into toluene (substrate) is carried out at the active-site Fe^{2+} iron atom resulting in the non-aromatic product (*cis*-dihydrodiol).

These enzyme systems have previously been classified in three groups, depending on subunit composition and redox centers (Batie *et al.*, 1991). Several genes coding for the enzymes performing this critical initial reaction have been cloned,

sequenced and characterized, and a number of crystal structures for the hydroxylase (Imbeault *et al.*, 2000; Karlsson *et al.*, 2003; Kauppi *et al.*, 1998) and also for the ferredoxin (Colbert *et al.*, 2000; Senda *et al.*, 2000), and reductase (Karlsson *et al.*, 2002) components have been resolved. Detailed investigations on a number of ring-activating dioxygenases, usually using chimeric proteins or directed or shuffled mutants, indicated the α subunit to be mainly, if not exclusively, responsible for substrate specificity (Beil *et al.*, 1998; Erickson and Mondello, 1993; Kimura *et al.*, 1997; Parales *et al.*, 1998; Tan and Cheong, 1994). Recent analyses have clearly shown that there is a clear discrepancy between the previous classification according to Batie *et. al* (Batie *et al.*, 1991) and the phylogenetic affiliation of the α-subunits determining substrate specificity (Nam *et al.*, 2001), and it was thus stated that the Batie system outlived its usefulness as a classification scheme for multicomponent non-heme iron oxygenases. Consequently, a new classification system based on the homology of the α-subunits of the terminal oxygenase components was proposed (Nam *et al.*, 2001). Actually, the oxygenase components can be considered to be a family with clearly identifiable subfamilies. In general, the clustering of oxygenases into families correlates with the native substrates oxidized by the members (Gibson and Parales, 2000). The toluene/biphenyl family includes enzymes for the degradation of toluene, benzene, isopropylbenzene, chlorobenzenes, and biphenyl. The naphthalene Family consists of enzymes for the degradation of naphthalene and phenanthrene but also for nitrobenzene and nitrotoluene degradation. The benzoate family is a diverse group consisting of enzymes that oxidize aromatic acids (benzoate, toluate, anthranilate, and isopropylbenzoate). Oxygenases of the toluene/biphenyl, naphthalene, and benzoate families catalyze dioxygenation reactions on their native substrates and are heteromultimers consisting of and α- and β-subunits.

Apart of the intrinsic importance of ring-hydroxylating dioxygenases for bioremediation (Dai *et al.*, 2002), oxygenases are of great interest because they carry out stereo- and regiospecific reactions (Seeger *et al.*, 2001) under mild conditions. This has led to their use as biocatalysts for the production of chemicals which were difficult or more expensive to produce by conventional methods such as indigo (Murdock *et al.*, 1993), 2,3-dihydroxybiphenyl, inositols, conduritols, acyclic sugars, and a wide range of biologically active products (Hudlicky *et al.*, 1999). Also the enantiospecificities of dioxygenases are interesting for production

of chiral chemical with industrial or medical applications (Boyd *et al.*, 2001; Gibson and Parales, 2000).

Because the aromatic ring hydroxylating dioxygenases have common evolutionary origins, they share consensus sequences, which can be recognized as gene family motifs. On the other hand, it was observed, that slight differences in amino acid sequence can be associated with dramatic changes in kinetic parameters like substrate specificity or regiospecificity (Beil, 1997; Erickson and Mondello, 1993; Parales *et al.*, 2000a). The generation of chimeric dioxygenase enzymes of *Burkholderia* sp. PS12 TecA tetrachlorobenzene dioxygenase (Beil, 1997) and *P.putida* F1 TodCBA toluene dioxygenase, allowed the identification of a single amino acid and of interacting regions essential for dechlorination of tetrachlorobenzene, and hybrid enzymes with extended substrate range acting on benzene and tetrachlorobenzene could be assembled (Beil *et al.*, 1998). Sequence differences which result in modified functions are mainly localized in positions close to active sites or channels, resulting in structural alterations of the encoded protein and the substrate binding pocket. To determine the relations between sequence differences and alteration in enzyme kinetic properties, resolved crystal structures are necessary to identify critical amino acids interacting with the substrate and forming the substrate binding pocket (Parales *et al.*, 2000a; Pollmann *et al.*, 2003). Based on the elucidated crystal structure (Kauppi *et al.*, 1998), position 352 of naphthalene dioxygenase of *Pseudomonas* sp. NCIB 9816-4 has been shown to modulate regioselectivity and enantioselectivity (Parales *et al.*, 2000b). Based on that crystal structure, amino acids important in regioselectivity and activity of tetrachlorobenzene dioxygenase of *Burkholderia* sp. PS12 have been identified (Pollmann *et al.*, 2003).

Further studies, evidencing the strong influence of single amino acid substitutions on kinetic properties of dioxygenases, have been performed using hybrid enzymes between biphenyl dioxygenases from *Burkholderia* sp. LB400 and *R. globerulus* P6 (Zielinski *et al.*, 2002). By creating and analyzing 23 independent single point mutations in biphenyl dioxygenase from LB400 (Zielinski *et al.*, 2003), severe changes in regiospecificity of substrate attack were often found to be associated with changes in amino acids located near to or in the substrate binding pocket, however, a considerable fraction of mutants exhibiting changes in regioselectivity carried mutations rather distant to the active site. These last findings emphasize that, even when a structural model is available, many protein substrate interactions can not be fully predicted.

A common theme in all these studies is, however, that slight sequence differences can heavily influence kinetic parameters. Respective information has mainly been recovered from cultured strains, a minor representation of the microbial diversity in the environment. First surveys on sequence and functional diversity of aromatic dioxygenases in environmental samples as analyzed by PCR amplification of genes of selected gene families, identified a very high sequence diversity in the non cultured fractions (Taylor *et al.*, 2002; Yeates *et al.*, 2000), and also new catalytic properties by complementation to partial known dioxygenase genes could be recovered from the natural diversity, again indicating that sequence changes altering kinetics would be tough to predict using structural models (Kahl and Hofer, 2003)

1.7.2 Ring hydroxylating monooxygenases

Alternative to dioxygenation of the aromatic ring, activation can be performed by monooxygenases. Various monooxygenases catalyzing monohydroxylation of hydroxyaromatics, and thus producing dihydroxyaromatics are single component flavoproteins (Harayama *et al.*, 1992). Salicylate hydroxylase is a flavoprotein monooxygenase that catalyzes the conversion of salicylate to catechol (Fig. 4). The enzyme was first purified from *P. putida* S1 (Yamamoto *et al.*, 1965), later from various other *Pseudomonas* and *Burkholderia* strains, and cloned and sequenced from various sources (Bosch *et al.*, 1999; Jones *et al.*, 2000). 4-Hydroxybenzoate-3-hydroxylase producing protocatechuate (Suarez *et al.*, 1995) or 3-hydroxybenzoate-6-hydroxylase producing gentisate (Wang *et al.*, 1987), dichlorophenol hydroxylases producing 3,5-dichlorocatechol (Farhana and New, 1997) and catechol producing phenol hydroxylase PheA from *Pseudomonas* sp. strain EST1001 (Nurk *et al.*, 1991) also belong to the single component flavoprotein monooxygenases. A chromosomal encoded flavoprotein phenol hydroxylase, designated TbuD, phylogenetically unrelated to PheA was described from *R. pickettii* PKO1 (Olsen *et al.*, 1997). However, also multicomponent phenol hydroxylases have been described (Shingler *et al.*, 1992) and the majority of phenol and methylphenol degrading organisms described thus far obviously use a multicomponent phenol hydroxylase. This is possibly due to their relatively broad substrate specificity, whereas PheA is restricted to transformation of phenol.

Multicomponent phenol hydroxylases, like monooxygenases catalyzing the hydroxylation of toluene and similar hydrophobic aromatics are members of the family of soluble diiron monooxygenases (Leahy *et al.*, 2003). These enzymes,

catalyzing the NAD(P)H-dependent hydroxylation (or epoxidation) of various hydrocarbons (Harayama *et al.*, 1992; Leahy *et al.*, 2003) are composed of three or four components: a hydroxylase protein, comprising two or three subunits in αβ or αβγ quarternary structure, a NADH-dependent oxidoreductase, a catalytic effector protein, and, occasionally, a ferredoxin with a Rieske iron centre (Colby and Dalton, 1978; Miura and Dalton, 1995; Newman and Wackett, 1995; Pikus *et al.*, 1996; Powlowski and Shingler, 1994; Small and Ensign, 1997) (Fig. 10).

The soluble diiron monooxygenases can be divided into four groups of enzymes based on their structure, and, to a lesser extent, their substrate specificity (Leahy *et al.*, 2003), the soluble methane monooxygenases, the *amo* alkene monooxygenase of *Rhodococcus corallinus* B-276, the phenol hydroxylases, and the four-component alkene/aromatic monooxygenases (Coufal *et al.*, 2000; Kahng *et al.*, 2001). These enzymes were in focus of research in the recent years because of their important catalytic activities and being involved in the carbon cycle of methan as a greenhouse gas and the transformation of pollutants such as benzene or tetrachloroethylene. The vast new information of their gene sequences, gene organization and substrate specificity, resulted in a better understanding of the enzyme determinants responsible for catalysis and of the gene family evolution (Leahy *et al.*, 2003). Phylogenetic analyses revealed that the α- and β-oxygenase subunits are paralogous proteins derived from an ancient gene duplication, with subsequent divergence yielding a catalytic α-oxygenase subunit and a structural β-oxygenase subunit. The oxidoreductase and ferredoxin components of these enzymes are likely to have been acquired by horizontal transfer from ancestors common to unrelated diiron and Rieske center oxygenases and other enzymes. The cumulative results of phylogenetic reconstructions suggest that the alkene/aromatic monooxygenases diverged first from the last common ancestor for these enzymes, followed by the phenol hydroxylases, Amo alkene monooxygenase, and methane monooxygenases. (Leahy *et al.*, 2003)

Of particular interest for environmental, ecological, and evolutionary issues, are those enzymes acting on aromatic compounds, (Fig. 10). They have been in the focus of several studies trying to unreveal the mechanism of the oxygenation catalytic cycle, to decipher determinants crucial for regiospecificity, production and transformation rates, and to modulate this reaction by protein engineering, to

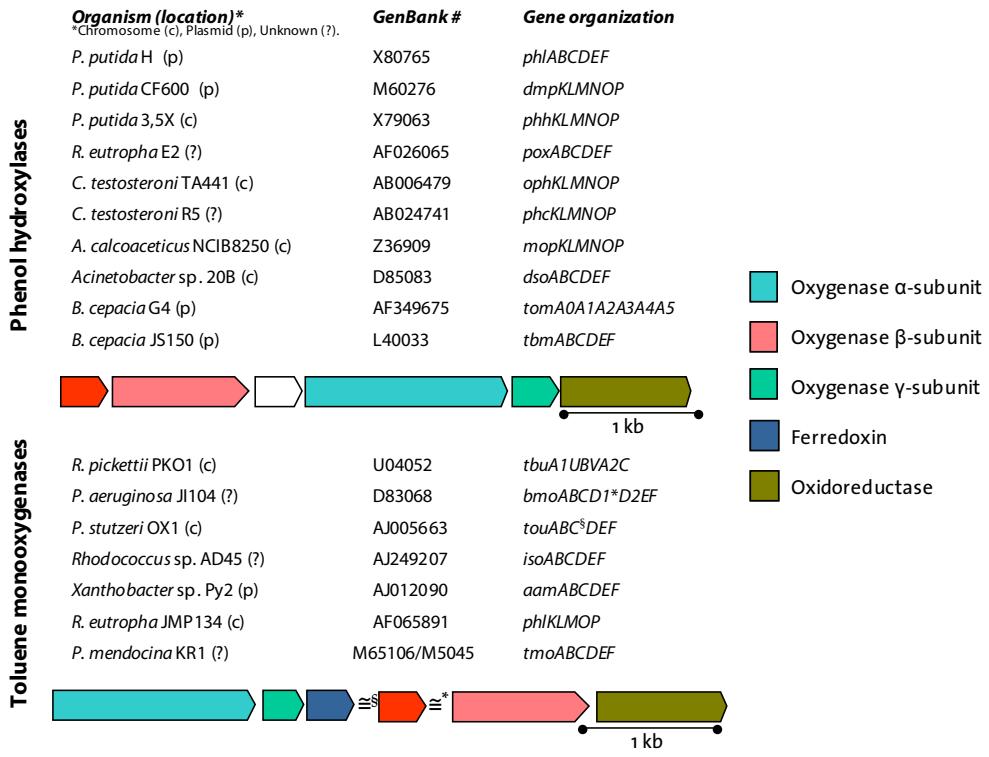

Fig. 10. Conserved gene arrangement in the clusters coding for aromatic ring hydroxylating monooxygenases. Phenol hydroxylase encoding operons are shown on the top, toluene/benzene monooxygenase encoding operons on the bottom. Intergenic lengths >200 bp (≠) in operons TOU and BMO, are indicated with, § and *, respectively.

improve the biotechnological performance in degradation of toxic pollutants like trichloroethylene or benzene. Members of two groups of soluble diiron monooxygenases, phenol hydroxylases and toluene/benzene monooxygenases, are known to act on aromatic pollutants.

1.7.2.1 Phenol hydroxylases

This group of enzymes consists of three-component oxygenases that share the capability to hydroxylate phenols to the respective catechols. Studies on the phenol hydroxylase of *P. putida* CF600 phenol hydroxylase (Dmp) (Nordlund *et al.*, 1990), and the toluene *o*-monooxygenase (Tom) of *B. cepacia* G4 (Newman and Wackett, 1995) have shown that these enzymes are composed of an $(\alpha\beta\gamma)_2$ diiron center hydroxylase system, an effector protein, and a FAD/$[Fe_2S_2]$ reductase (Fig. 10). Other members of this phylogenetic group are the phenol hydroxylases of *P. putida* H (PhlD) (Herrmann *et al.*, 1995), *P. putida* 3,5X (PhhN) (Ng *et al.*, 1994), *Ralstonia* sp. E2 (PoxD) (Hino *et al.*, 1998), *Ralstonia* sp. KN1 (PhyC) (Ishida and Nakamura, 2000), *Comamonas testosteroni* strains TA441 (AphN) (Arai *et al.*, 1998) and R5 (PhcN) (Teramoto *et al.*, 1999), cresol monooxygenase (CrpD) (Byrne *et al.*, 1995) of *R. picketii* PKO1 and toluene monooxygenases Tbc1D and TbmD (Johnson

and Olsen, 1995; Kahng *et al.*, 2001), of *Burkholderia* sp. JS150 (Johnson and Olsen, 1995; Kahng *et al.*, 2001). Whereas all these monooxygenases share the capability to hydroxylate phenol and methyl substituted derivatives, only a few enzymes of this group are capable to hydroxylate the uninactivated benzene nucleus. These enzymes, among them Tom, sequentially oxidize toluene to 2-methylphenol and further to 3-methylcatechol (Newman and Wackett, 1995). Even though broad specificity phenol hydroxylases cannot, thus far, be differentiated from narrow substrate specificity hydrolase based on sequence motifs or phylogeny, their kinetic properties in phenol turnover correlate with phylogenetic relationships (Futamata *et al.*, 2001).

1.7.2.2 The four component alkene/aromatic monooxygenases

The four component alkene/aromatic monooxygenases, are composed of a $(\alpha\beta\gamma)_2$ diiron hydroxo-bridged center hydroxylase, a Rieske-type ferredoxin, a NADH reductase and an effector protein (Fig. 10). Reported members of this group of monooxygenases include (the designation of gene encoding the the α-subunit is given in parenthesis) *R. pickettii* PKO1 toluene 3-monooxygenase (TbuA1) (Olsen *et al.*, 1994), *P. stutzeri* OX1 toluene/*o*-xylene monooxygenase (TouA) (Bertoni *et al.*, 1998), *R. eutropha* JMP134 phenol hydroxylase (PhlK) (Kim *et al.*, 1996), *B. cepacia* JS150 toluene/benzene 2-monooxygenase (Tbc2A) (Olsen *et al.*, 1997), *B. cepacia* AA1 toluene/benzene hydroxylase (TbhA) (Ma and Herson, 2000), *P. mendocina* KR1 toluene monooxygenase (TmoA) (Whited and Gibson, 1991b), but also the alkene monooxygenase of *Xanthobacter* sp. strain Py2 (Small and Ensign, 1997) and isoprene monooxygenase of *Rhodococcus* sp. strain AD45 (van Hylckama Vlieg *et al.*, 2000). As alkene monooxygenase of strain Py2 has been observed to also hydroxylate benzene and toluene (Zhou *et al.*, 1999), the capability to hydroxylate the unactivated benzene nucleus seems to be a common property of this group of enzymes. Some of the enzymes of this subfamily, like the *tou* gene products of strain OX1 (Bertoni *et al.*, 1998), have also been shown to oxidize phenol and methylphenols, whereas others do not harbor this capability (Whited and Gibson, 1991b; Yen *et al.*, 1991). Beside their difference in substrate specificity these enzymes also differ in their regioselectivity. Whereas the Tbu toluene 3-monooxygenase produces dominantly 3-methylphenol (Olsen *et al.*, 1994), the archetypal Tmo toluene 4-monooxygenase of *P. mendocina* KR1 produces dominantly 4-methylphenol (Whited and Gibson, 1991a). In last mentioned enzyme it was shown that the effector protein not only is necessary for effective coupling,

but also for a high regioselectivity (Mitchell *et al.*, 2002). Whereas a complete monooxygenase system produced nearly exclusively 4-chlorophenol from chlorobenzene (Pikus *et al.*, 1997), absence of the effector protein resulted in a significant formation of 2-chloro- and 3-chorophenol (Mitchell *et al.*, 2002). However, the α-subunit was found to be dominantly responsible for regioselectivity and a G103L mutant was shown to produce nearly equal amounts of 2-chloro-, 3-chloro-, and 4-chlorophenol. The product distribution of the mutant enzyme from toluene was similar to that observed in other naturally occurring members of the toluene/benzene hydroxylase branch of the diiron hydroxylase phylogenetic tree such as *P. aeruginosa* JI104 (Kitayama *et al.*, 1996) and *P. stutzeri* OX1 (Bertoni *et al.*, 1996). The organization of the operons coding for these enzymes is well conserved, but not their localization, which can be on plasmids or on chromosomes (Fig. 10). It is commonly encountered that these operons are localized close to operons coding for enzymes of *meta*-pathways, which are transforming intermediate catechols into Krebs cycle intermediates (see Figs. 4 and 8).

1.7.3 Aromatic ring cleavage dioxygenases

A common step in the different pathways for aerobic monoaromatic degradation was early identified as the ring fission of the hydroxylated catecholic metabolites (Dagley *et al.*, 1960; Davies and Evans, 1964; Hayaishi and Hashimoto, 1950; Hayaishi and Stanier, 1951; Ornston and Stanier, 1964), opening the ring and producing the respective cleavage products which are further metabolized to Krebs cycle intermediates, thereby, allowing the mineralization of the pollutant. Enzymes catalyzing such reactions comprise catechol, protocatechuate, gentisate, homoprotocatechuate and homogentisate dioxygenases, among others. Depending on the mode of ring cleavage, the catechol dioxygenases as well as the protocatechuate dioxygenases can be classified into two major functional groups, the intradiol dioxygenases, cleaving between the hydroxyl substituents (*ortho*-cleavage, catalyzed by catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases) (Ornston, 1966b) and the extradiol dioxygenases cleaving beside one of the hydroxyl substituents (*meta*-cleavage, catechol 2,3-dioxygenases and protocatechuate 4,5- or 2,3-dioxygenases) (Broderick, 1999; Noda *et al.*, 1990; Orville *et al.*, 1997; Que and Ho, 1996; Wolgel *et al.*, 1993). The degradation of the most prominent environmental pollutants benzene, toluene and the xylene isomers usually proceeds via catechols as central intermediates, and the degradation of

toluene via protocatechuate has thus far only been observed in *P. mendocina* KR1 (Whited and Gibson, 1991b).

1.7.3.1 Catechol 1,2-dioxygenases

The catechol 1,2-dioxygenases are a group of dioxygenases cleaving catechol between the hydroxyl groups (Harayama *et al.*, 1992; Ornston, 1966a) (Fig. 11). The catechol 1,2-dioxygenase contain non-heme non-iron-sulfur Fe (III) as a prosthetic group (Hayaishi *et al.*, 1957), and a substrate activation mechanism has been proposed (Nishida *et al.*, 1992). These enzymes have been in focus of research interest because of their environmental importance, as they perform a crucial step in the degradation of aromatics metabolized to catecholic intermediates. In *Pseudomonas*, the genes encoding the catechol 1,2-dioxygenase (CatA) and the two enzymes (CatBC) that transform the ring-cleavage product, *cis,cis*-muconate, into 3-oxoadipate enol-lactone (the common intermediate of catechol and protocatechuate degradation) are localized in a single gene cluster (Harwood and Parales, 1996). Conversion of the latter to Krebs cycle intermediates is carried out by the products of the *pcaD*, *pcaIJ* and *pcaF* genes involved also in protocatechuate degradation (Jimenez *et al.*, 2002) (Fig. 11).

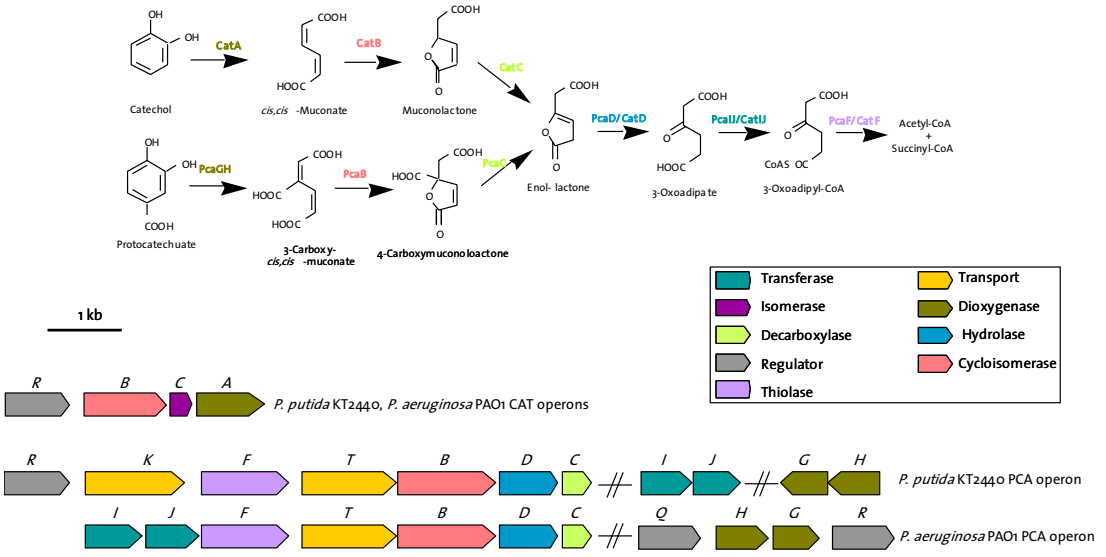

Fig. 11. Metabolic pathways of catechol and protocatechuate via intradiol cleavage and operon arrangement in two *Pseudomonas* sp. genomes. Catechol and protocatechuate branches of the 3-oxoadipate pathway. The organization of gene clusters for protocatechuate *(pca)* and catechol *(cat)* metabolism in *P. putida* KT2440 (Jimenez *et al.*, 2002; Nelson *et al.*, 2002) and *P. aeruginosa* PAO1 (Jimenez *et al.*, 2002; Stover *et al.*, 2000) are shown. *catA*, catechol 1,2-dioxygenase gene; *catB*, muconate cycloisomerase gene; *pcaB*, carboxymuconate cycloisomerase gene; *catC*, muconolactone isomerase gene; *pcaC*, carboxymuconolactone decarboxylase gene; *catD* and *pcaD*, 3-oxoadipate enol-lactone hydrolase genes; *catF* and *pcaF*, thiolase genes; *pcaHG*, protocatechuate 3,4-dioxygenase genes; *pcaIJ* and *catIJ*, transferase genes; *pcaK* and *pcaT*, 4-hydroxybenzoate and dicarboxylate transport protein genes; *pcaR* and *pcaQ*, regulator genes.

In some bacterial species, such as *Acinetobacter* sp. ADP1, the catechol branch and the protocatechuate branch never converge and two independently regulated set of genes encode isofunctional enzymes for the last three steps of the pathway (Harwood and Parales, 1996). Some microorganisms have been reported to contain two gene copies encoding catechol 1,2-dioxygenases (Jimenez *et al.*, 2002). Most of the catechol 1,2-dioxygenases reported are homodimers of identical subunits, however, in case of two copies of catechol 1,2-dioxygenase encoding genes, active homo- and heterodimers can be formed (Nakai *et al.*, 1990). Catechol 1,2-dioxygenases are usually of relaxed substrate specificity, and capable of tranforming methyl substituted derivatives (Dorn and Knackmuss, 1978; Murakami *et al.*, 1997). However, whereas catechol is usually transformed to Krebs cycle intermediates by enzymes of the 3-oxoadipate pathway, methylcatechols are only cometabolized, and methyl-substituted muconolactones are formed, which are not further transformed by the 3-oxoadipate pathway enzymes (Catelani *et al.*, 1971; Knackmuss *et al.*, 1976). Thus, only poor information is available about the further fate of those compounds (Erb *et al.*, 1998; Pieper *et al.*, 1985; Prucha *et al.*, 1997). A special group of intradiol cleavage enzymes, only distantly related to catechol 1,2-dioxygenases, and based on its even broader substrate specificity termed chlorocatechol 1,2-dioxygenases, is capable of transforming chlorocatechols at high rates (Broderick and O´Halloran, 1991; Dorn and Knackmuss, 1978; Pieper *et al.*, 1988). Due to their potential to mineralize chlorinated aromatic pollutants, they have been observed to be suitable targets for DNA-based methods to study environmental samples (van der Meer, 1997; van der Meer *et al.*, 1998).

1.7.3.2 Extradiol dioxygenases

Extradiol dioxygenases have been reported to be involved in various aromatic degradative pathways, and are mechanistically characterized by their capability to introduce both atoms of dioxygen into their substrates, resulting in a ring-cleavage *meta* to the hydroxyl groups. Those enzymes comprise catechol 2,3-dioxygenases, 2,3-dihydroxybiphenyl dioxygenases, protocatechuate 4,5- and 2,3-dioxygenases and 3,4-dihydroxyphenylacetate dioxygenases among others. A non-heme Fe(II) (or exceptionally Mn(II) (Boldt *et al.*, 1995)) present in the active site is needed as prosthetic group to accomplish the ring fission. Comparison of sequence data (Eltis and Bolin, 1996) indicate that there are at least two types of extradiol dioxygenases, each having originated from distinct predecessors. The majority of extradiol dioxygenases reported thus far belong to the so-called type I

dioxygenases, which all contain the PROSITE consensus sequence (Eltis and Bolin, 1996). Crystal structures of several extradiol dioxygenases have been resolved (Eltis *et al.*, 1993; Han *et al.*, 1995; Kita *et al.*, 1999; Sato *et al.*, 2002; Senda *et al.*, 2000; Sugimoto *et al.*, 1999; Uragami *et al.*, 2001; Vetting and Ohlendorf, 2000), showing that they can form mono-, di-, tri-, tetra-, or octamers.

To classify this group of functionally similar enzymes (extradiol dioxygenase family), their evolutionary relationships were investigated (Eltis and Bolin, 1996). Usually, these enzymes are composed of homologous N- and C-terminal domains (two domain enzymes), each of which is constructed from two βαβββ motifs. Thus, this family examplifies the diversification of proteins by single gene duplication (Lo Conte *et al.*, 2002)

Phylogenetic analyses clearly showed that enzymes having a preference for monocyclic substrates (catechol 2,3-dioxygenses) and those showing a preference for bicyclic aromatics (2,3-dihydroxybiphenyl dioxygenases), form two separate clades. A third clade is formed by single domain homologues (Asturias and Timmis, 1993; Heiss *et al.*, 1997). Regarding the degradation of monocyclic aromatics, family I.2, comprising most of the enzymes described thus far with a preference for monocyclic catechols, can be regarded as of special interest. Most currently known members of this family fall into a single cluster, termed subfamily I.2.A, a cluster of closely related enzymes with activities against monocyclic catecholic derivatives (Fig. 12).

1.7.3.3 Catechol 2,3-dioxygenases subfamily I.2.A.

Studies on enzymes in this subfamily have received special attention for many reasons. On one hand, these enzymes seem to be widely distributed in soil bacteria (Nakazawa, 2002), secondly, they catalyze a key metabolic step common to the degradation of a broad range of aromatic pollutants, and third, this activity can be easily screened for by the production of the yellow coloured ring-cleavage product (2-hydroxymuconic semialdehyde) from catechol. Several bacterial strains from all around the world exhibiting this activity have been isolated, and this activity is strongly linked to their capabilities to mineralize aromatic compounds. The respective genes can be found in natural hydrocarbon degraders from soil (Cavalca *et al.*, 2000; Joshi and Walia, 1996), or even in more extreme environments like deep anoxic hypersaline lakes (Brusa *et al.*, 2001).

The formation of a coloured reaction product enables the rapid functional screening for the presence of such genes in genomic libraries, allowing the

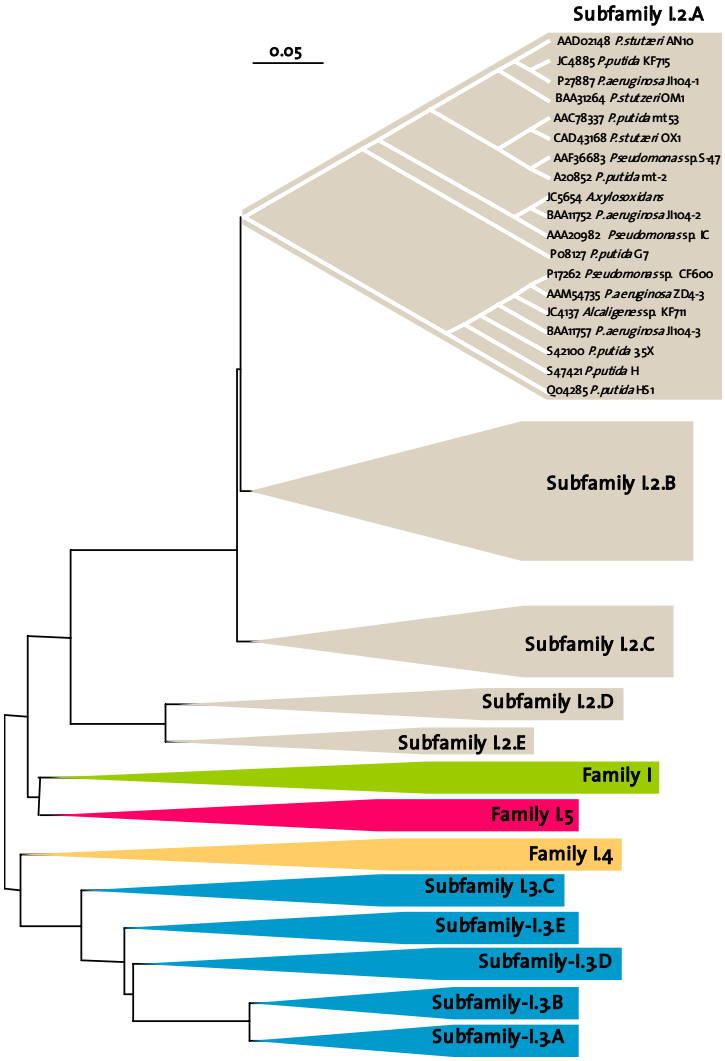

Fig. 12. Phylogenetic tree of extradiol dioxygenases, and the cluster defining subfamily I.2.A according to Eltis and Bolin (1996).

selection of positive clones, the sequencing of the genes, overexpression of the proteins and detailed studies of enzyme activities. In addition, the phylogenetic relationship permits the use of consensus sequence information to detect, by PCR with primers able to comprise and amplify all the known members of this subfamily, new polymorphisms in gene fragments of environmental origin.

Several studies used the C23O genes as targets to assess catabolic potential in autochthonous environmental bacteria. Several supporting evidences showed that the PCR detection or the increment in gene copies of C23O genes is linked to increased aromatic levels in the environmental samples the microbial community is being analysed (Brusa *et al.*, 2001; Cavalca *et al.*, 2000; Hallier-Soulier *et al.*, 1996; Joshi and Walia, 1996; Mesarch *et al.*, 2000; Vacca *et al.*, 2002; Wikstrom *et al.*, 1996). These findings indicate that strains with C23O activity are selected when the

environment is challenged with increasing levels of aromatic pollutants. While many studies focus on the detection of C23O genes by PCR, the sequence diversity inside the amplified fragments is usually not assessed. The reported C23O genes from isolates exhibit variation in their sequences, and such variations may have implications on enzyme structure and, thus, activity. Only a few reports are available on elucidating differences in substrate specificity of extradiol dioxygenases (Mars *et al.*, 1999; McKay *et al.*, 2003; Riegert *et al.*, 2001; Vaillancourt *et al.*, 2003), however, significant differences in kinetic properties of single amino acid mutants (Cerdan *et al.*, 1995; Cerdan *et al.*, 1994) or between subfamily I.2.A enzymes were observed (Kitayama *et al.*, 1996), which even resulted in different growth phenotypes (Cerdan *et al.*, 1994). The knowledge on sequence diversity and structure/function relationships in C23O genes can be expanded by recovering natural variants, however, thus far, only one study reported on the analysis of hybrid C23O proteins containing C23O fragments amplified from polluted environments (Okuta *et al.*, 1998). Microdiversity inside this C23O subfamily resulting in different substrate specificities or activities has an important meaning in the context of flux of metabolites, as the lack of efficient degradation of a particular catecholic metabolite could affect the cell growth cell and survival. Accumulation of catechols is known to have acute toxic and even lethal effects to bacteria, decreasing the overall biodegradation rate of the aromatic (Perez-Pantoja *et al.*, 2003; Schweigert *et al.*, 2001). Therefore, a detailed knowledge of C23O sequence determinants responsible for discrete substrate specificities and enzyme kinetics would be very helpful for a rapid environmental diagnosis. With this information and by applying molecular techniques to analyse the non-culturable fraction, it seems possible to go beyond the mere detection of C23O fragments, to fine levels of its sequence information, and to analyze adaptation processes in the environment.

1.8 Metabolism of substrate mixtures

Pollutants in the environment never occur as single pollutants, but nearly always as pollutant mixtures. One of the most prominent environmental pollutants is benzene, which is always found together with toluene, and xylenes, and often ethylbenzene. Most studies in the laboratory analyzed single strains and their capabilities to mineralize these compounds when present as single substrates. However, a careful assessment of the biochemistry of the different metabolic pathways described already shows, that the degradation of pollutant mixtures, like

BTEX, necessitates the presence of a fine tuned net of metabolic interactions (Chang *et al.*, 2001; Deeb and Alvarez-Cohen, 1999). As an example, microorganisms harbouring a dioxygenolytic pathway are usually capable to degrade benzene and toluene, whereas xylenes are usually not mineralized. This is due to two metabolic problems (Fig 13). *o*-Xylene and *m*-xylene are subject to monooxygenation by toluene dioxygenases, resulting in the formation of the respective methylsubstituted benzylalcohols (Lehning *et al.*, 1997; Pollmann *et al.*, 2001). *p*-Xylene is transformed to 3,6-dimethylcatechol. Further metabolism of methylsubstituted benzylalcohols necessitate the presence of a TOL type pathway (Shaw and Harayama, 1990). Both benzylalcohol and benzylaldehyde dehydrogenases are of broad substrate specificity capable to transform all methylsubstituted derivatives leading to the corresponding benzoates, with the exception of 2-methylbenzaldehyde, which is not a substrate for benzaldehyde dehydrogenase (Shaw and Harayama, 1990). Moreover, despite its simple structure, the metabolic fate of 2-methylbenzoate is not clear. 2-Methylbenzoate is only a poor substrate for toluate dioxygenase (Ge *et al.*, 2002), and thus far only one organism capable to degrade 2-methylbenzoate has been described (Higson and Focht, 1992).

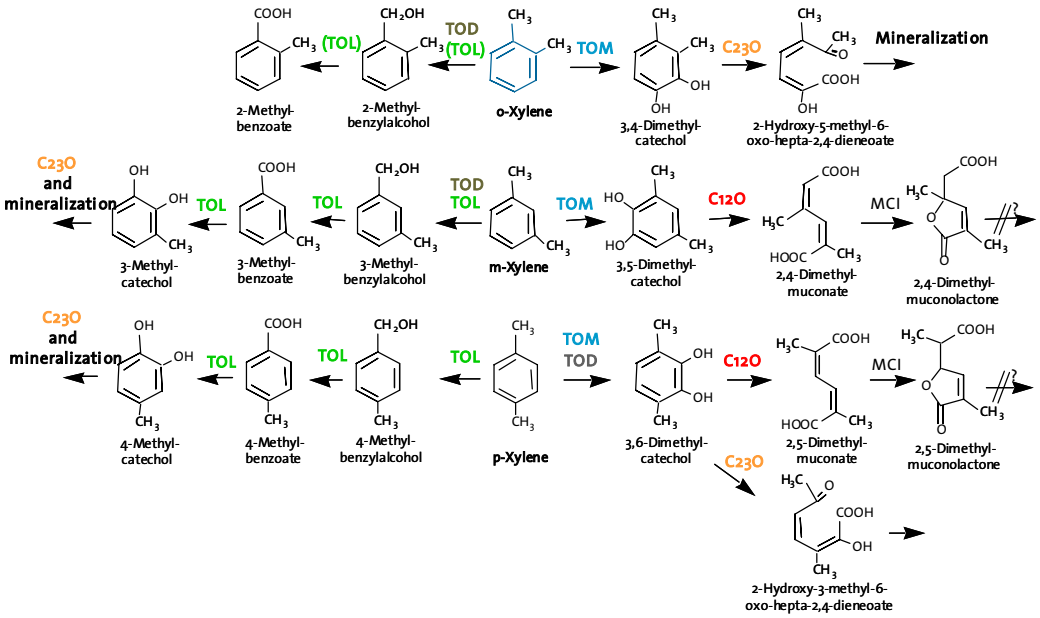

Fig. 13. Diversification in routes to degrade xylenes. TOL, TOD and TOM paths. Metabolism of xylenes by the TOL and the TOD pathway and by microorganisms catalyzing successive monooxygenations (TOM, toluene monooxygenase pathways). The TOL pathway is suited for degradation of m- and p-xylene, but not for o-xylene. The TOD pathway is suited for none of the xylenes, and the TOM pathways for degradation of only o-xylene. C23O, catechol 2,3-dioxygenase; C12O, catechol 1,2-dioxygenase; MCI, muconate cycloisomerase.

The TOL pathway is suited for the degradation of toluene, *m*- and *p*-xylene. *o*-Xylene is not transformed by xylene monooxygenase of the TOL pathway (Abril *et*

al., 1989; Brinkmann and Reineke, 1992). A monooxygenase pathway is necessary to achieve *o*-xylene degradation (Bertoni *et al.*, 1996), and such a pathway is definitely suited to mineralize toluene and even benzene. However, in case of metabolism of *m*- and *p*-xylene, 3,5- and 3,6-dimethylcatechol are formed (Fig. 13). Whereas 3,4-dimethylcatechol, the oxidation product of o-xylene, is easily degraded by *meta*-cleavage pathways (Barbieri *et al.*, 2001; Favaro *et al.*, 1996; Kunz and Chapman, 1981), there are no reports on the mineralization of 3,5- or 3,6-dimethylcatechol via the *meta*-cleavage pathway. In the *o*-xylene degrader *P. stutzeri* OX1, none of these catechols is a substrate for C23O (Barbieri *et al.*, 2001). Even if so, 3,5-dimethylcatechol can not be mineralized, as action of 2-hydroxy-2,4-dienoate hydratase is prevented by the methylfunction in the tentative intermediate (2-hydroxy-4-methyl-2,4-dieneoate). Also, C23O enzymes of *Ralstonia eutropha* JMP134 were not capable of transforming 3,5-dimethylcatechol, whereas poor activity against 3,6-dimethylcatechol was evidenced (Pieper *et al.*, 1995). In contrast, these catechols were subject to slow intradiol cleavage giving rise to dimethylmuconolactones as dead-end products.

It is thus evident, that metabolism of simple compound mixtures like BTEX under environmental conditions is by far not understood yet. Further developments to retrieve information on biodegradative pathways and networks from microbial communities are still needed. This could be achieved by molecular methods, like detection, quantification and fingerprinting of catabolic genes. It would help to discern first the catabolic genes and catabolic routes with higher fitness when communities are confronted with pollutant mixtures followed by analysis of predominant polymorphisms

1.9 Objectives

The main objective of this experimental work was to develop and apply new molecular techniques in order to characterize the genetic potential, catabolic structure and degradative activities against BTEX in microbial communities from aquifers and their adaptation to organic contaminants. To achieve these goals, the criteria to select a model ecosystem, molecular techniques, and gene targets, take into consideration the understanding of the whole adaptation process as an evolutionary event, where the contaminant is the selection pressure, and the predominance of certain phenotypes, gene families, or gene polymorphisms, are biological fitness factors.

1.9.1 Model ecosystem

As a model ecosystem to follow adaptation of natural communities to aromatic contamination, an area with a long history of BTEX (benzene, toluene, *o*-, *m*-, *p*-xylenes and ethylbenzene) contamination located in Litninov, Czech Republic has

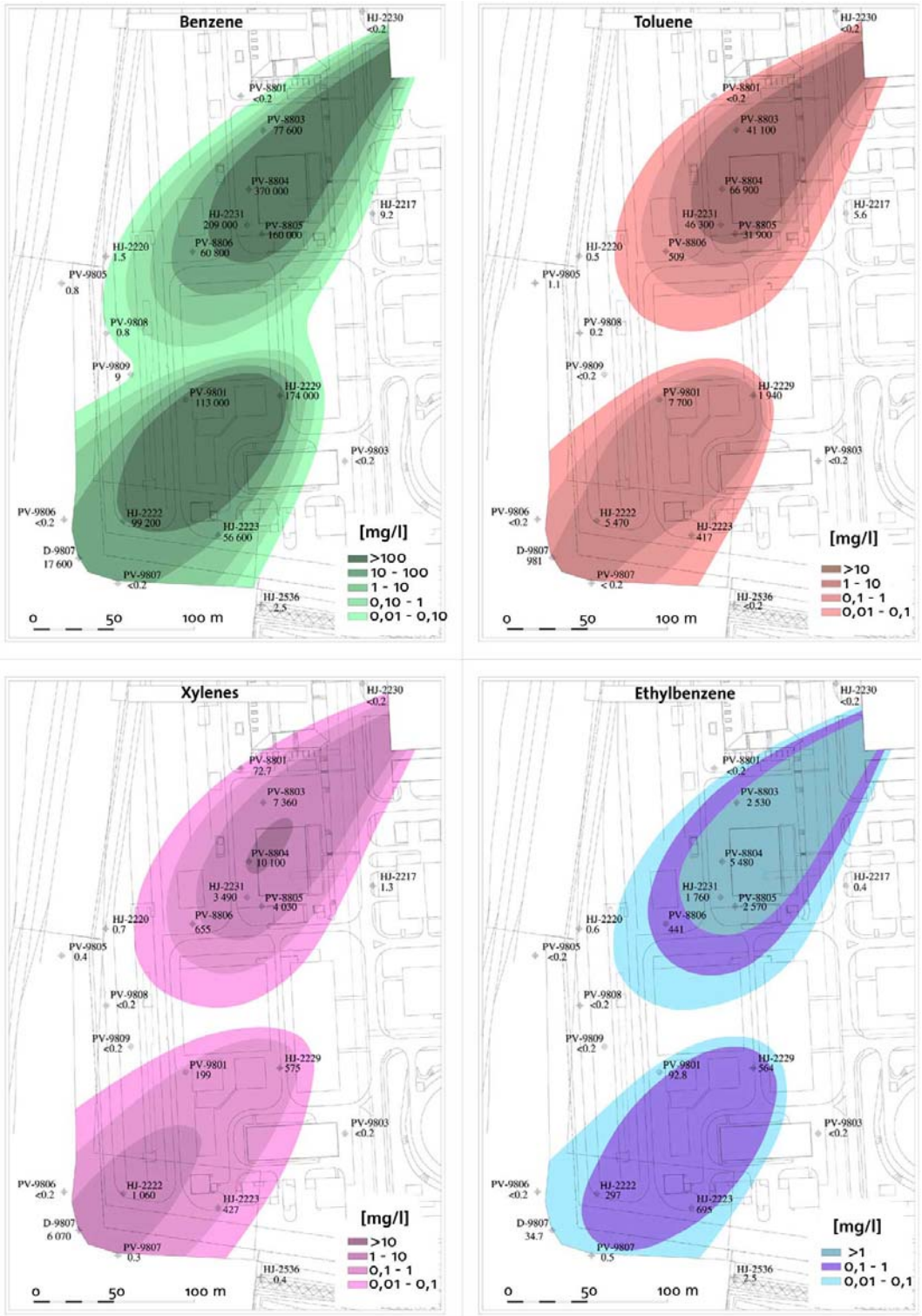

Fig. 14. Distribution of single BTEX compounds in the model ecosystem

been selected in the frame of the AMICO EC-project (QLK3-CT-2000-00731). To understand this contamination plume, soils and groundwater from this area have been subject to hydrogeological studies. Chemical and physical analysis had been performed by AQUATEST a.s., an ISO 9001 accredited Czech company. This company has been modeling the dynamics of the main underground BTEX plumes, finding sources, directions of groundwater flow and movement of the pollutant plume, and concentrations of BTEX gradients.

The pollution profiles of the plume are shown in figure 14. The concentrations of benzene in the highly contaminated area are very high, in the range of 100 mg/l. The detailed plume characterization allowed the selection of soil sampling points with different degrees of contamination with an homogenous soil composition background, ranging from virtually clean soils with no detectable BTEX concentrations, to very high BTEX contaminated spots. Soils that became contaminated during the course of the project were also included in the microbial analyses.

1.9.2 Molecular fingerprints to follow gene diversity in communities

Catabolic genes can be used for a genetic assessment of biodegradation potential, the intrinsic functional potential a microbial community has to degrade aromatic pollutants. Targeting different gene families can give an overview of the metabolic net acting in biodegradation *in situ*. Changes in diversity and predominant polymorphisms in given gene families can give indications on functional adaptations, as the modified amino acid positions are possibly selected for optimal functioning under environmental conditions. Thus, quick and reliable comparisons of predominant polymorphisms and changes in diversity of functional genes between different samples are a way to recover natural catabolic diversity, and to predict metabolic performance.

Genes coding for critical steps in aromatic degradation such as ring activation or cleavage, are ideal targets to follow biodegradation of aromatics in the environment. Regarding biodegradation of non-chloro-substituted monoaromatics, genes coding for catechol 2,3-dioxygenases (C23O), used in several studies as molecular targets in sites with ongoing bioremediation activities, and to assess metabolic potential, are ideal catabolic indicators to be used for the optimisation of molecular fingerprint methods targetting functional genes. They are widely distributed in many environments, and C23O gene copies are known to increase

under increased aromatic contamination. However, it is not known if and how C23O gene diversity changes under pollutant stress.

One of the main purposes of this experimental work was to develop the necessary means to be able to detect shifts in diversity of functional genes. The methods were developed using C23O genes as targets. To analyse the culturable fractions, new isolation procedures based on expression of C23O activity rather than growth were performed. To analyse the non culturable fraction, C23O molecular fingerprints from environmental DNA were optimised. DNA sequencing and activity analyses of predominant variants by cloning and recombinant expression assured the functional meaning of the sequence information retrived from the environment.

This Doctoral Thesis will show experimental evidences supporting usefulness of molecular methods to assess aromatic catabolic potential and adaptations of microbial communities by analyzing C23O functional diversity. Additionally, catabolic targets to optimize and expand this strategy will be suggested.

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CHAPTER II

Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria[§]

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Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria

2.1 Abstract

To determine phylogenetic diversity of a functional gene from strain collections or environmental DNA amplifications, new and fast methods are required. Catechol 2,3-dioxygenase (C23O) subfamily I.2.A genes, known to be of crucial importance for aromatic degradation, were used as a model to adapt the amplified ribosomal DNA restriction analysis to functional genes. Sequence data of C23O genes from 13 reference strains, representing the main branches of the C23O family I.2.A phylogeny were used for simulation of theoretical restriction patterns. Among other restriction enzymes, *Sau*3A1 theoretically produce characteristic profiles from each subfamily I.2.A member and their similarities reassembled the main divergent branches of C23O gene phylogeny. This enzyme was used to perform an amplified functional DNA restriction analysis (AFDRA) on C23O genes of reference strains and 19 isolates. Cluster analyses of the restriction fragment profiles obtained from isolates showed patterns with distinct similarities to the reference strain profiles, allowing to distinguish 4 different groups. Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the AFDRA approach. AFDRA thus provided a quick assessment of C23O diversity in a strain collection and insights of its gene phylogeny affiliation among known family members. It can not only be easily applied to a vast number of isolates but also to define the predominant polymorphism of a functional gene present in environmental DNA extracts. This approach may be useful to differentiate functional genes also for many other gene families.

2.2 Introduction

It is well documented that only a small fraction of environmental microorganisms can be cultured to date and, thus, culture independent methods (Amann *et al.*, 1995; Staley and Konopka, 1985) to describe environmental microbial community composition, to detect new community members, activities and functions, have experienced a fast development in the last decades. Culture independent approaches, usually focused on 16S rRNA phylogeny, have been applied for analysing populations in diverse environments (Torsvik and Ovreas, 2002), and various molecular fingerprinting methods like denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA), terminal restriction fragment length polymorphism (T-RLFP), upstream-independent ribosomal RNA amplification analysis (URA) and polymerase chain reaction - single strand conformation polymorphism (PCR-SSCP) have been optimised to obtain a fast and reliable overview on microbial community compositions and their shifts by changing environmental conditions (Kent and Triplett, 2002). Other fingerprinting methods like amplified ribosomal DNA restriction analysis (ARDRA) (Khetmalas *et al.*, 2002) or PCR - multiple enzyme restriction fragment length polymorphism (PCR-MERFLP) (Porteous *et al.*, 2002) have been developed to distinguish single 16S rDNA PCR fragments from isolates or gene libraries to get a rapid overview on taxonomical diversity in cultivable or non cultivable microbial fractions.

However, a number of bacterial genes such as those involved in antibiotic resistance, antimicrobial production or pollutant degradation, are selected or present independently of the rate of evolution and taxonomy of the bacterial host (Davison, 1999). Thus, assessment of community functions needs reliable tools to analyse those functions rather than taxonomical composition. PCR based techniques have been used to detect functional/catabolic genes in environmental isolates or environmental DNA, and diversity is usually assessed by sequencing of genes from isolates or PCR clone libraries (Buchan *et al.*, 2001; Duarte *et al.*, 2001; Hamelin *et al.*, 2002; Yeates *et al.*, 2000). Recent reports showed that PCR amplified fragments of catabolic genes from environmental DNA can be separated by DGGE (Henckel *et al.*, 1999; Nicolaisen and Ramsing, 2002), and RFLP analyses were used to select distinctive restriction patterns of single amplicons for further sequence determinations (Bakermans and Madsen, 2002; Braker *et al.*, 2000; Yan *et al.*, 2003). A more detailed knowledge on catabolic genes, retrieved by culture independent methods and from isolates, can significantly improve our

understanding of microbial functioning and degradation processes in the environment, which would help to design new bioremediation strategies (Widada *et al.*, 2002). However, no fast and reliable tools are available to rapidly analyse gene phylogeny in respective culture collections or to determine abundant gene variants and their phylogeny in the environment.

Catechol 2,3-dioxygenases (C23O) comprise a family of genes coding for a group of enzymes with aromatic ring fission activity, which play an essential role in the degradation of a wide range of aromatic pollutants. A large collection of genes coding for such an activity have been cloned and sequenced in the last years, being classified as a diverse gene family (Eltis and Bolin, 1996). The gene phylogeny of these very closely related sequences does not follow strictly a taxonomical relation with the bacterial hosts, since these genes are mainly found on plasmids, and their evolution and conservation rates are heavily affected by traits like selection pressures, horizontal transfer and mobile genetic elements (Williams *et al.*, 2002). Conserved regions of the C23O genes have been selected in several studies as suitable PCR targets in culture independent approaches, showing a correlation in relative C23O abundances dependent of pollutants levels (Erb and Wagner-Dobler, 1993; Mesarch *et al.*, 2000; Meyer *et al.*, 1999; Ringelberg *et al.*, 2001; Wikstrom *et al.*, 1996).

In this study we developed and evaluated an amplified DNA restriction analysis targeting a functional gene (AFDRA) in a collection of strains sharing a phenotypic character, *meta*-cleavage activity. We examined the suitability of this method to directly analyse soil DNA, by a combination of PCR serial dilution assays and AFDRA analyses. This approach allowed us to determine C23O gene copy numbers and predominant C23O gene variants in soil samples.

2.3 Material and Methods

2.3.1 Microorganisms, samples, isolation and culture conditions

Reference strains *P. stutzeri* AN10 (Bosch *et al.*, 2000), *P. putida* CF600 (Bartilson and Shingler, 1989), *P. putida* G7 (Ghosal *et al.*, 1987), *P. putida* H (Herrmann *et al.*, 1995), *Pseudomonas sp.* IC (Carrington *et al.*, 1994), *P. aeruginosa* JI104 (Kitayama *et al.*, 1996), *Sphingomonas sp.* KF711 (Moon *et al.*, 1996), *P. putida* mt-2 (Nakai *et al.*, 1983), *P. putida* mt53 (Keil *et al.*, 1985), *P. stutzeri* OM1 (Ouchiyama *et al.*, 1998), *P. stutzeri* OX1 (Arenghi *et al.*, 2001), *P. putida* HS1 (Benjamin *et al.*, 1991), *P. putida* 3,5X (Hopper and Kemp, 1980) and isolated bacterial strains were cultured on R2A agar (DIFCO) at 30°C for 3 days.

Bacterial strains were isolated by direct plating on R2A agar of appropriate soil dilutions from samples obtained from four sampling points of a BTEX contaminated area in Czech Republic. After 3 days of incubation at 30°C, colonies were tested by spraying with a 100 mM aqueous catechol solution, and those exhibiting *meta*-cleavage activity, indicated by yellow coloration of the medium, were selected, streaked and purified.

For culture independent analyses, soil samples were collected from the capillary fringe zone (zone of essentially water saturated soil just above the water table) and the saturated zone (zone below the water table) of a highly contaminated area (BTEX concentrations in the groundwater of (320, 97, 6 and 13 mg/l of benzene, toluene, ethylbenzene and xylenes respectively, as determined by Aquatest a.s. Prague). Total DNA was extracted in triplicates from 500 mg of each soil sample, and purified with the Fast Prep Soil DNA Extraction Kit (BIO101). Concentration of DNA was determined by using PicoGreen dsDNA Quantitation Kit (Molecular Probes) and a microtiter plate reader as described previously (Weinbauer and Höfle, 2001).

2.3.2 Primer design and PCR conditions

The primers C23O-ORF-F 5'AGG TGW CGT SAT GAA MAA AGG 3' and C23O-ORF-R 5' TYA GGT SAK MAC GGT CAK GAA 3' were designed to amplify 934-bp comprising the complete open reading frames of the subfamily I.2.A C23O genes. Degenerations are placed according to all the possible variable positions in the alignment of 20 C23O reference sequences (EMBL/ GenBank/ DBBJ accession numbers M33263, AY112717, S77084, X80765, D83057, X77856, M65205, X60740, JC4885, AF039534, AB001722, V01161, AF226279, AF102891, AJ496739, D83042,

JC5654, U01825, AY228547, X06412). As template DNA, 4 µl aliquots of a total of 50 µl supernatant from colonies boiled for 10 min (Kanakaraj *et al.*, 1998) or 5 µl of serial dilutions of soil DNA (prepared from 50 µl of a total DNA extraction of 500 mg of soil) were applied in a PCR mixture containing 1x PCR Buffer (Promega) supplemented with 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotide triphosphate, 0.25 µM of each primer (synthesized by Invitrogen) and 0.3 U/µl *Taq* DNA Polymerase (Promega).

For amplification of soil DNA, PCR was performed in a PCRExpress gradient thermocycler (Hybaid) as follows: an initial step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 90 s. For amplification of DNA from isolates, the PCR program comprised an initial step at 95°C for 3 min, 10 cycles of denaturation at 94°C for 45 s, annealing for 45 s at a starting temperature of 60°C with a decrease in annealing temperature of 1°C per cycle, elongation for 90 s at 72°C, followed by 25 PCR cycles at a constant annealing temperature of 55°C, final elongation step at 72°C for 8 min and further storage of the reactions at –20°C.

To determine the minimum number of C23O copies detected by PCR with this set of primers, an equimolar mixture of 15 C23O PCR amplicons, (from 13 reference strains and from isolates number 9 and 19), was serially diluted and aliquots of these dilutions applied in triplicates to PCR reactions. The maximum dilution at which a PCR signal of the expected size was detected in one of the triplicates was determined analysing 3 µl of the PCR reactions by gel electrophoresis (1.5% agarose, 10 cm length, 1X TAE running buffer, 1 hour at 95 volts and visualized by ethidium bromide staining) (Sambrook *et al.*, 1989). As the average molecular mass of the 934-bp C23O fragments is 570 kDa, it can be calculated that a single PCR amplicon molecule corresponds to approximately 9.5×10^{-10} ng of DNA. The minimum number of C23O copies necessary to produce a detectable PCR amplification with the primers assayed, was used to extrapolate the number of copies per milligram of soil (Ringelberg *et al.*, 2001), by determination of the maximum dilution of soil DNA at which a PCR signal is detected in 6 independent experiments. The inhibitory effect of soil DNA extracts on C23O PCR amplification efficiency was determined by spiking soil DNA (5 µl containing a total of 10 ng of DNA) extracted from a control sample of the saturated zone of the study area with no previous BTEX contamination and avoid of amplifiable C23O genes, with 1 ng of an equimolar mixture of 15 C23O PCR amplicons (equivalent to 10^7 C23O gene copies). By serial dilutions in triplicates, the maximum dilution at which a C23O PCR signal of the expected size is detected was determined.

2.3.3 In silico PCR-RFLP analyses

Predictions and simulations of the restriction fragment length of 13 C23O DNA sequences from reference strains was performed by a restriction mapping software (Heiman, 1997) with around 300 different endonuclease recognition sites. Enzymes that potentially produce phylogenetic informative fragments were selected after detailed inspection and comparison of the predicted restriction positions and calculation of the produced fragments lengths. Matrices to represent discrete values for presence or absence of a predictable restriction fragment size in the sequence dataset were generated for each selected enzyme. Distance estimations on these matrices were performed with Treecon software (Van de Peer and De Wachter, 1993) by Simple Matching or by Nei and Liu methods. Tree topology was inferred by the UPGMA clustering method. Those results were compared with C23O protein phylogeny.

2.3.4 AFDRA (Amplified functional DNA restriction analysis)

Selected restriction enzymes (NEB) were used in reactions in a final volume of 20 µl containing the 1X buffer recommended by the manufacturer, 3 U of the enzyme, and approximately 200 ng of PCR product, incubated at optimal temperature for 4 hours.

The restriction fragments patterns were resolved by gel electrophoresis in a 5.5% Nusieve 3:1 (FMC Bioproducts) agarose matrix (14 cm x 11cm) in 1X TBE buffer (Sambrook *et al.*, 1989), at 120V (80 mA), until the bromophenol blue dye in the loading buffer was reaching the front edge (approximately 5 h). The loading buffer comprised glycerol (30%, v/v) in water, xylene cyanol and bromophenol blue at final concentrations of 0.025%. To correctly detect all bands and intensities onto the gel, the loading buffer contained dyes at 1/10 strength of the standard concentrations (Sambrook *et al.*, 1989), avoiding their collateral effect of dark background. For staining, the gel was placed in ethidium bromide solution (10 mg/l) for 30 minutes. The gel was visualised, images acquired and stored on a gel documentation system (Vilber Lourmat).

2.3.5 DNA sequencing and phylogenetic analyses

Nucleotide sequencing of PCR fragments or plasmids with cloned inserts was carried out on both strands using Taq dye-deoxy terminator in an ABI 373A automatic DNA sequencer (Perkin-Elmer Applied Biosystems) following the protocols provided by the manufacturer. Primers used for sequence reactions were the same as for PCR. Alignments were performed with CLUSTAL X 1.8 windows

interface of the CLUSTALW program using default values (Thompson *et al.*, 1997). DNA alignments were edited and translated with the GeneDoc program (Nicolas, 1997). Phylogenetic trees were obtained with the option available on the CLUSTAL program through the Neighbour Joining (N-J) algorithm method. Distances were generated using Kimura Matrix, and tree stability was supported through Bootstrap analysis. NJ trees were visualized with the NJplot program (Perriere and Gouy, 1996). Values of more than 50% of 1000 replications (seed value 111) are shown on appropriate branches. A similarity matrix to compare PCR-RFLP gel patterns was calculated using Bio1D v.99.02 Software (Vilber Lourmat) with Jaccard coefficients with 3% confidence applied to both compared bands. A dendrogram of similarity values in the matrix was calculated using the UPGMA algorithm. The sequences of gene fragments reported in this study are available under the EMBL/ GenBank/ DBBJ accession numbers AJ544921 to AJ544938.

2.4 Results and Discussion

2.4.1 Targeting the functional gene by PCR

From soil samples contaminated with BTEX, a broad set of microorganisms exhibiting catechol 2,3-dioxygenase activity, identified based on a simple test for induction of this enzyme activity during growth on agar plates, was isolated. All isolates referred here could use benzene and/or toluene as sole source of carbon and energy.

We hypothesized that these isolates could carry C23O genes members of the I.2.A subfamily (Eltis and Bolin, 1996), because this group of enzymes is known to be involved in the degradation of aromatic compounds in several environmental strains. A primer set was designed to amplify the complete C23O gene of members in the I.2.A subfamily, annealing at the start and stop codon positions. For the forward primer, annealing was optimised by placing its 5' side 10 bases upstream of the C23O starting codon. This primer set was tested on 13 reference strains (see material and methods) known to carry C23O subfamily I.2.A gene variants and on a soil bacterial strain collection. From all the reference strains and from 30 out of 37 bacterial isolates tested, a signal of the expected size was successfully amplified, whereas 7 isolates did not give any amplification product, indicating that they probably harbour a C23O of another subfamily. 19 of those isolates showing an amplification product of 934-bp in size were randomly selected for further analysis.

2.4.2 Selection of restriction enzymes producing patterns clustering as the divergent branches of C23O protein phylogeny by using predictions and simulations on reported DNA sequences

To assess the diversity of the C23O genes, a fast screening method to identify potential redundant or highly similar genes based on a restriction fragment simulation was developed. This method was compared with results obtained by a conventional nucleic acid sequencing of the genes. The theoretical simulation was performed through the generation of discrete matrices of restriction fragments sizes produced in C23O sequences deposited on EMBL/GenBank/DDBJ databases. These matrices were further analysed to find restriction enzymes producing specific fragment profiles for different genes, and a restriction pattern related to gene phylogeny. In this search, out of approximately 300 enzymes tested on each of the 13 selected sequences, some enzymes, among them *Alu*I, *Bsi*HKAI, *Bst*UI,

*Hha*I, *Hin*P1I, *Msp*R9I, *Pal*I, *Sau*3AI and *Taq*I, were found as theoretically producing characteristic profiles from each subfamily I.2.A member.

2.4.3 Characterization and comparison of C23O diversity in bacterial isolates by AFDRA and DNA sequencing

After a meticulous comparison of the predictions, we selected the restriction enzyme *Sau*3A1 for the experiments. This enzyme could cleave the C23O genes in such a way that the resulting clustering of the matrix (37 x 11), representing discrete values for presence or absence of a predictable restriction fragment size (37 theoretical restriction fragments from 11 reference strains. Fig. 1), reassembled the main divergent branches of the C23O phylogeny (Eltis and Bolin, 1996). Furthermore, the predicted fragment size range produced by this enzyme (10 – 447 bp) could be separated by high resolution agarose gel electrophoresis. *Sau*3A1 restriction assays were performed using PCR C23O gene amplifications from 7 reference strains (Fig. 2) selected to cover the main divergent branches of the protein gene phylogeny. Comparisons of the restriction atterns under optimised electrophoretic conditions resolved the informative restriction fragment patterns of reference strain amplifications in the >35-bp to <600-bp range, effectively discriminating down to 5-bp size differences, as evidenced by the comparison of 133-bp and 138-bp fragments. The same procedure was performed on 19 PCR amplified products of bacterial isolates, which were producing distinctive restriction fragment patterns. Digestions were usually complete under the conditions used and, only in case of high concentrations of amplification product subjected to digestions, faint bands due to incomplete digests were observed.

Cluster analyses of the restriction fragment profiles obtained allowed to distinguish 4 groups of restriction patterns present in the isolates. A selection of those representative patterns found with distinct similarities to the reference strain profiles are shown in Fig. 2. The most dominant pattern, observed in 13 of 19 isolates of C23O PCR-RFLP, was equal to that obtained from *P. stutzeri* AN10. Another common pattern, which shared some restriction fragment sizes with *P. putida* G7, was present in 4 isolates. The PCR fragment from a single isolate produced a restriction pattern closely related to that from *P. putida* mt-2. Another PCR fragment from a single isolate ->

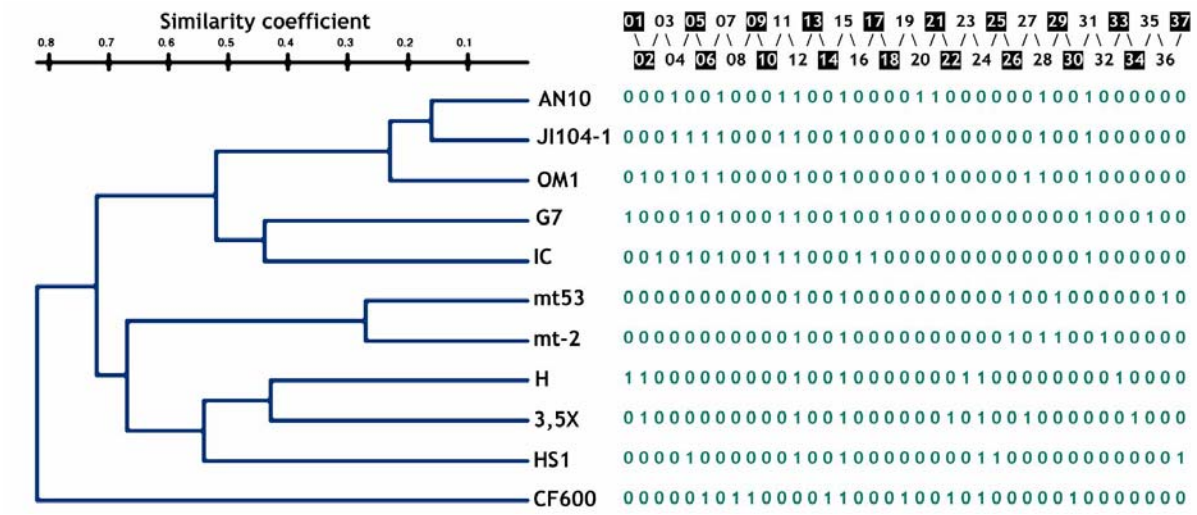

Fig. 1. Cluster dendogram of theoretical *Sau3*AI restriction fragments produced from C23O genes of *P. stutzeri* AN10, *P. putida* CF600, *P. putida* G7, *P. putida* H, *Pseudomonas sp.* IC, *P. aeruginosa* JI104, *P. putida* mt-2, *P. putida* mt53, *P. stutzeri* OM1, *P. putida* HS1, and *P. putida* 3,5X. The corresponding discrete matrix of presence (1) or absence (0) of a specific restriction fragment size is given to the right. Columns 1 to 37 represent, respectively, the following sizes in base pairs: 10, 14, 18, 19, 21, 23, 30, 34, 35, 39, 44, 55, 58, 88, 92, 104, 111, 112, 126, 133, 138, 141, 152, 153, 156, 157, 163, 174, 177, 232, 239, 269, 285, 306, 321, 443, 447.

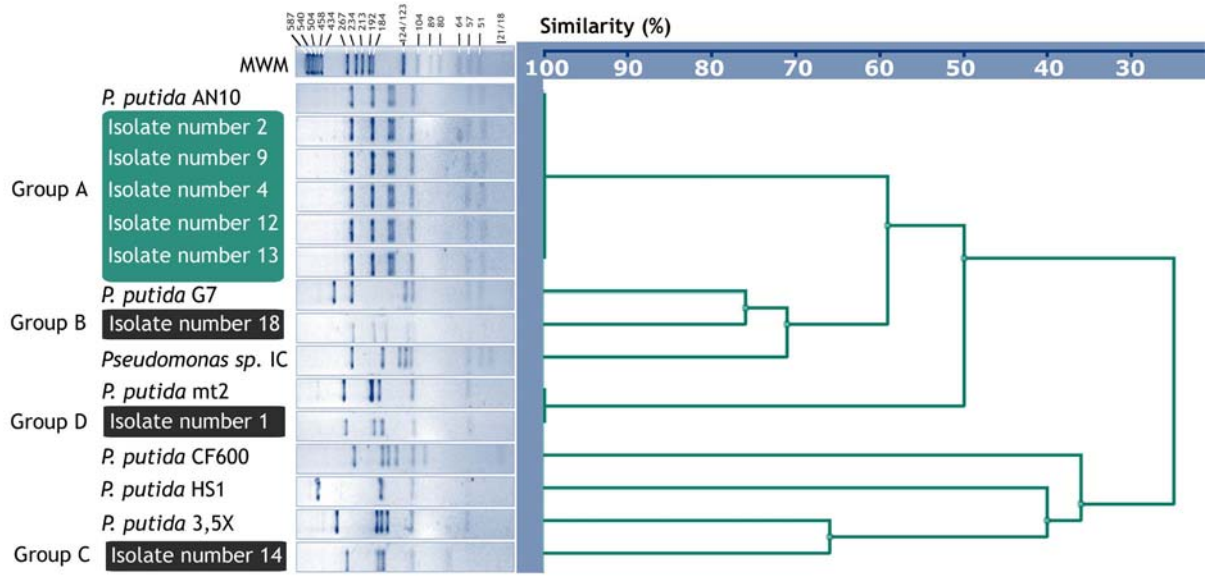

Fig. 2. Agarose gel-generated cluster dendogram illustrating the relationship of C23O genes based on similarity of restriction fragments patterns produced by *Sau3*AI digestions. Digestions of the 934-bp C23O PCR fragments amplified from the reference strains *P. stutzeri* AN10, *P. putida* CF600, *P. putida* G7, *Pseudomonas* sp. IC, *P. putida* mt-2, *P. putida* HS1, *P. putida* 3,5X, and isolated bacterial strains of Group A: isolates number 2, 9, 4, 12 and 13, Group C: isolate number 14, Group B: isolate number 18 and Group D: isolate number 1. Marker lane (MWM) shows DNA marker V (Roche), in the upper side are written the corresponding base pair length values of each DNA fragment.

showed a restriction pattern sharing, to a lesser extent, band sizes with the *P. putida* 3,5X C23O gene. It can thus be hypothesized, that the isolates are dominated by catechol 2,3-dioxygenase genes closely related to the *P. stutzeri* AN10 gene, whereas other isolates harbour far divergent evolutionary variants of catechol 2,3-dioxygenases.

To confirm the reliability of this assay, the fragments from all the isolates mentioned above were completely sequenced, and the resulting sequences were aligned against C23O DNA sequences of reference strains. In the phylogenetic tree of the deduced C23O protein sequences from reference strains and isolates (Fig. 3), the sequences obtained from isolates are clustering in 5 different branches. This closely corresponds to the results obtained by the restriction analysis. In addition, all the sequences that were supposed to carry the same or a closely related gene polymorphism, are in fact, at sequence level sharing this feature. (Fig. 2 and Fig. 3). In the case of Group A, comprising 13 isolates, a single point mutation difference is present, producing a difference in the coding of the amino acid at position 218 (referred to *P. putida* mt-2 C23O protein numbering) to tyrosine (11 isolates, subgroup A_1) or histidine (2 isolates, subgroup A_2) (Fig. 3). This difference does not affect a *Sau*3AI recognition site and therefore could not be discriminated by PCR-RFLP. In such cases, where small differences like point mutations should be detected, a screening technique not retrieving gene phylogeny information but with higher sensitivity for single nucleotide substitutions like SSCP (Orita *et al.*, 1989) or DGGE (Muyzer *et al.*, 1993) could be then applied.

2.4.4 AFDRA of new C23O sequences follow the expected relation with gene phylogeny

The PCR-RFLP discrete matrix (40 x 18) of C23O members, including the 4 new sequences observed in isolates and comprising three new PCR-RF sizes only present in these isolates is shown in Fig. 4. The thereby generated dendrogram reassemble the main divergent clustering topology of the C23O protein phylogeny (Fig. 3). The application of PCR-RFLP in a closely related group of sequences diminishes the potential limitations of the RFLP technique (Hillis *et al.*, 1996), as neither larger insertion/deletions or rearrangement events nor the convergent fragments, were observed in the sequences analysed. However, the cluster analysis of the bands resolved on the gel showed an outgrouping restriction pattern of the *P. putida* CF600 C23O ->

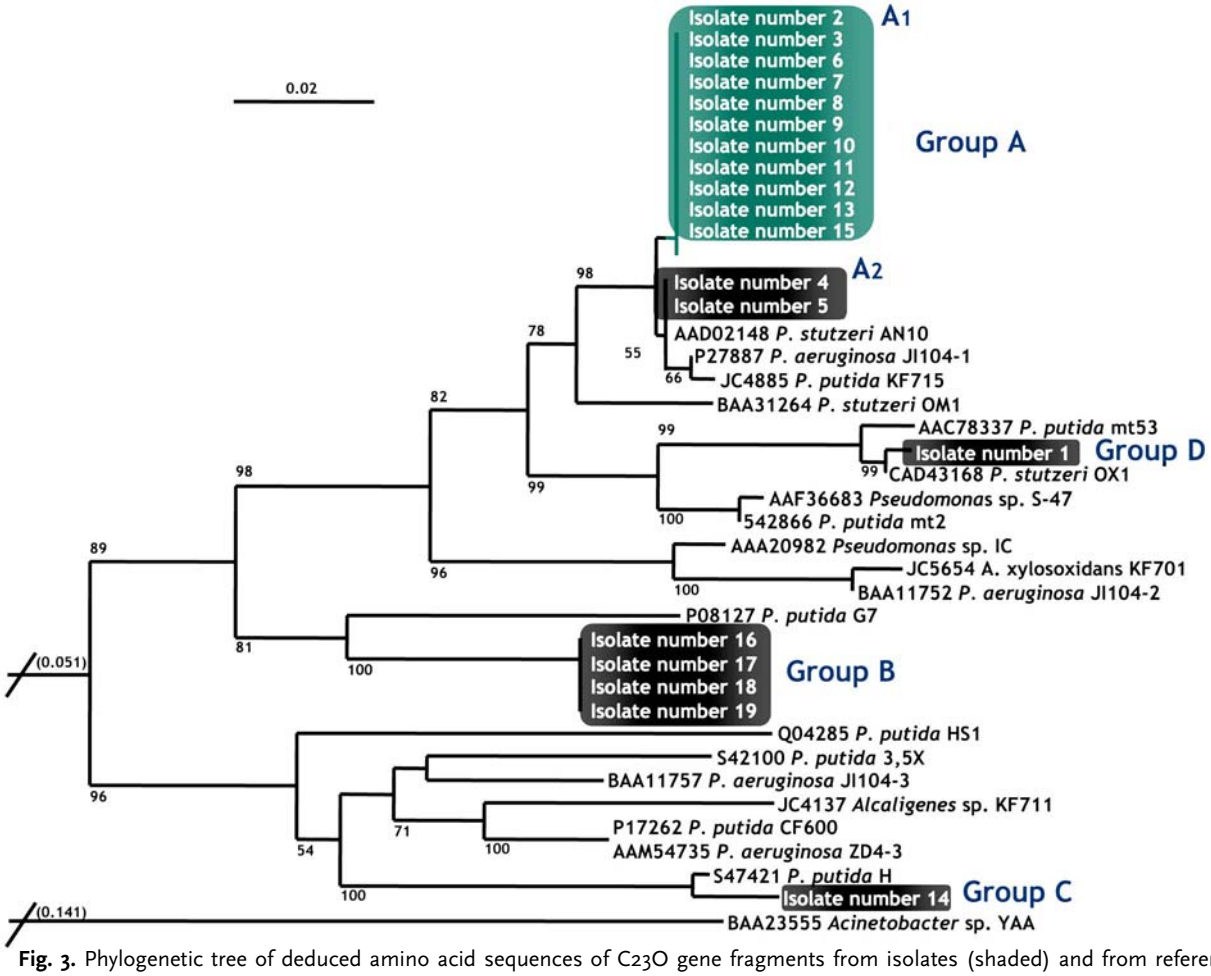

Fig. 3. Phylogenetic tree of deduced amino acid sequences of C23O gene fragments from isolates (shaded) and from reference strains (indicated by their EMBL/GenBank/DDBJ accession number, abbreviated organism name and strain designation). Alignment of a 268 amino acid length block was performed with CLUSTAL W using default values. N-J phylogenetic tree was generated using the option available in CLUSTAL W program. Bootstrap values above 50% from 1000 neighbor-joining trees are indicated to the left of the nodes. Bar represents 2 amino acid changes per 100 amino acids.

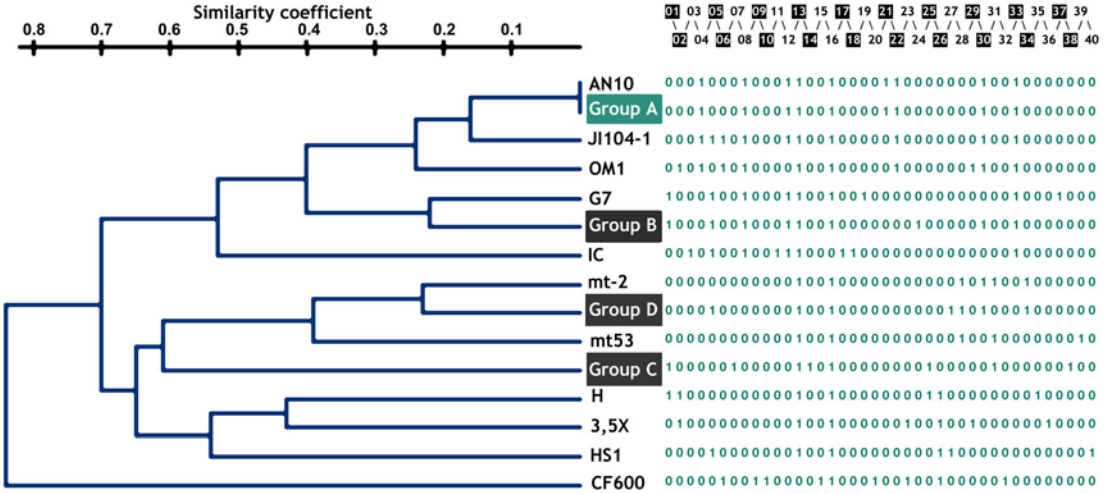

Fig. 4. Cluster dendogram and corresponding discrete matrix of presence-absence of a restriction fragment size of theoretical restrictions of C23O genes with *Sau*3AI from reference strains and isolates. Reference strains are *P. stutzeri* AN10, *P. putida* CF600, *P. putida* G7, *P. putida* H, *Pseudomonas sp.* IC, *P. aeruginosa* JI104, *P. putida* mt-2, *P. putida* mt53, *P. stutzeri* OM1, *P. putida* HS1, *P. putida* 3,5X. Group A includes sequences from isolates number 2, 9, 4, 12 and 13, Group C includes isolate number 14, Group B includes isolate number 18 and Group D includes isolate number 1. The resultant 40 columns represent the following sizes in base pairs (1 to 40 respectively): 10, 14, 18, 19, 21, 23, 27, 30, 34, 35, 39, 44, 55, 58, 88, 92, 104, 111, 112, 126, 133, 138, 141, 147, 152, 153, 156, 157, 163, 174, 177, 232, 239, 269, 285, 306, 321, 353, 443, 447.

gene, and its 141-bp fragment was incorrectly assigned as clustering with fragments corresponding to 138-bp. That is an expected limitation of the technique due to the experimental error in calculation of very similar fragment sizes. Nevertheless, the generation and detection of a high number of discrete characters allowed an approximated reconstruction of gene phylogeny. AFDRA proved thus to be a valuable tool to screen PCR fragments of functional genes from isolates, avoiding the redundant work of repeated sequencing of the same gene from different isolates or colonies, saving time and resources, and giving a fast and reliable overview of the predominance and phylogenetic affiliation of the genes in a group of environmental bacterial isolates. It can be particularly useful in studies determining functional gene diversity in collections of numerous environmental isolates, as well as to screen PCR fragments of functional genes amplified from environmental DNA.

2.4.5 Quantitative and qualitative applications of the AFDRA on soil DNA

The efficiency and the minimum amount of C23O copies detected with our primers designed to target the C23O subfamily I.2.A were determined, as a prerequisite for a quantitative and qualitative application of AFDRA to DNA extracted from soil. PCR products were observed in all triplicates when the PCR mixture contained 10^{-8} ng of DNA (corresponding to approximately 10 template molecules) and usually in one of the triplicates when it contained 10^{-9} ng of DNA (corresponding to approximately 1 template molecule), independent of the complexity of the applied experimental C23O gene composition (Fig. 5.A). Soil DNA extractions yielded 100 to 300 ng of DNA per gram of soil for the samples analysed. C23O PCR products were observed from as low as 20 pg of soil DNA as template (Fig. 5.B.), indicating that 1 g of soil of the sampling point analysed would contain at least 10^4 C23O gene copies, independent if the soil was collected from the capillary fringe or the saturated zone. However, a control soil DNA extract avoid of C23O genes and spiked with 10^7 C23O gene copies showed an amplification signal only, when the serial dilution contained at least 100 copies of C23O genes. Thus, it can be assumed that the soil extract decreased the C23O amplification efficiency by a factor of 100. This in turn indicates that the contaminated soils analysed would contain approximately 10^6 C23O copies per gram of soil. To determine if the amplification affects the diversity recovered, AFDRA was applied on the PCR products obtained from artificial C23O gene mixtures containing 10 - 10^4 gene copies.

The primers did not show evidence for preferential amplification of any specific polymorphism under the conditions tested (Fig. 5. C; lanes 17 and 18). The pattern obtained from an equimolar mixture of 15 C23O gene variants was very complex and did not reassemble clearly any of the single C23O gene profiles, even when the applied template mixture contains only approximately 10 C23O copies. On the other hand, the primers amplified preferentially the most abundant polymorphism out of a complex template mixture containing a 10-fold excess of the *P. putida* CF600 C23O gene, (Fig. 5. C. lanes 19, 20 and 26). These results show the potential use of AFDRA to rapidly determine the predominant gene polymorphism in complex environmental DNA mixtures. When AFDRA was applied to PCR products obtained from 200 pg of soil DNA, characteristic patterns were observed. When PCR-amplified DNA extracted from the capillary fringe zone was analysed (lane 21) a characteristic pattern identical to that obtained from *P. putida* AN10 (lane 25) or Group A-isolates was observed. This result perfectly match with the information obtained by screening of a PCR clone library constructed with a 527-bp C23O fragment amplified from this soil sample (Junca and Pieper, unpublished). The C23O gene composition in the saturated zone differed qualitatively from that of the capillary fringe horizon (Fig. 5.C. lane 23), evidencing the coexistence of two most abundant C23O polymorphisms: one closely related to Group C-isolates and another equal to that obtained from *P. putida* AN10 - Group A-isolates.

2.4.6 Concluding remarks

In this report we demonstrated that the ARDRA approach can be transferred to assess functional diversity of environmental isolates in a very convenient first selection providing preliminary phylogenetic information, for further focused and dedicated biochemical characterization, point mutation determinations or sequencing work, instead of random or massive screenings. The method proved to be also appropriate, coupled to a quantitative survey, to assess predominance of gene variant(s) in DNA extracted directly from environmental samples. Future works will determine if the technique can be optimised to refine its phylogenetic resolution power, by additional testing of multiple restrictions of a single PCR fragment (Porteous *et al.*, 2002) or by integrated comparisons of profiles obtained in independent restrictions with different enzymes (Sikorski *et al.*, 2002).

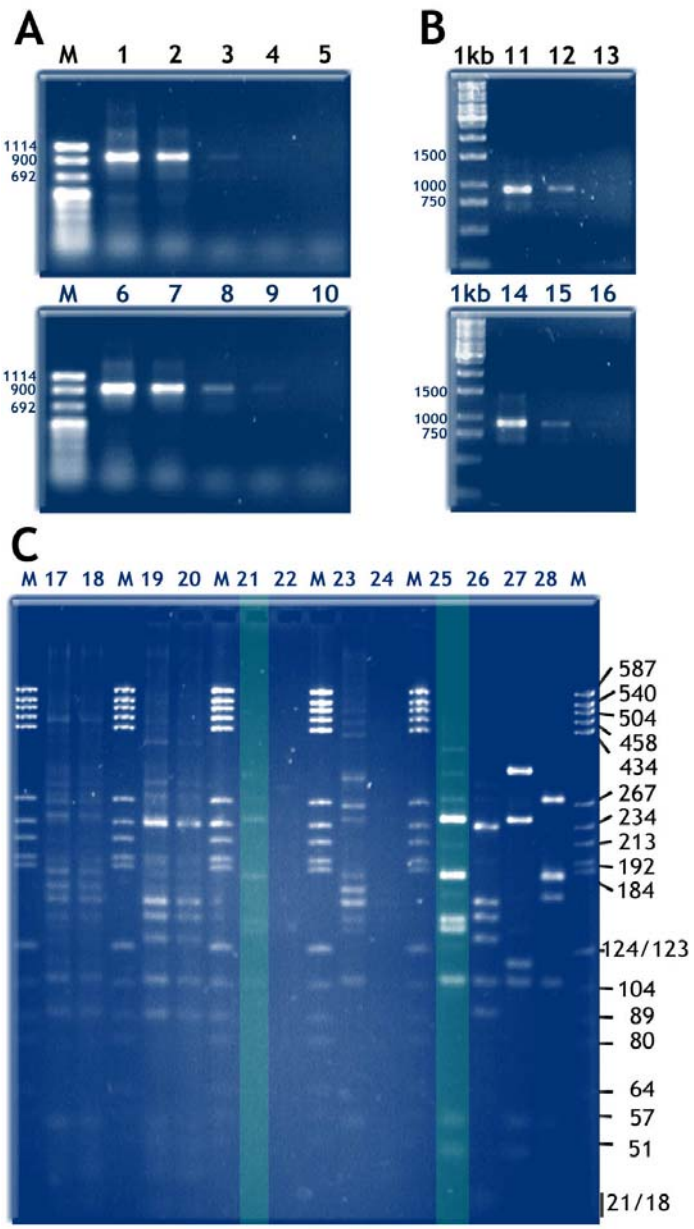

Fig. 5. Detection limit, diversity comprised, and performance in artificial mixtures or environmental DNA of the primers targeting C23O subfamily I.2.A and assessment of predominant gene variants in soil DNA by AFDRA approach.

A. Detection limit of primers designed to target C23O subfamily I.2.A. Lanes 1 to 5 show PCR products obtained from 10^{-6}, 10^{-8}, 10^{-10}, $10^{-11}$and 10^{-12} ng, respectively, of an equimolar mixture of 15 C23O genes. Lanes 6 to 10 show PCR products obtained from 10^{-5}, 10^{-7}, 10^{-9}, 10^{-10} and 10^{-11} ng, respectively, of an equimolar mixture of 14 C23O gene variants supplemented with a 10-fold excess of the *P. putida* CF600. Lanes M were loaded with 200 ng of DNA Molecular Weight Marker VIII (Roche).

B. Determination of the C23O copy number in contaminated soils. Total DNA extracted from 500 mg of soil (100 ng) was resuspended in 50 µl of H_2O with a DNA concentration of 2 ng/µl, and appropriate dilutions subjected to PCR amplification. PCR products obtained from DNA extracted from a soil sample of the capillary fringe zone (lanes 11 – 13) or from the saturated zone (lanes 14 - 16) were analysed by agarose gel electrophoresis. PCR products correspond to those obtained from 200 pg, 20 pg or 2 pg of soil DNA, respectively. Lanes 1kb were loaded with 100 ng of GeneRuler 1kb DNA ladder (MBI Fermentas).

C. AFDRA patterns obtained from PCR-amplified artificial C23O mixtures or direct amplifications of soil DNA. Lanes 17 and 18 show AFDRA of the PCR amplification products obtained from 10^{-6} or 10^{-8} ng of an equimolar mixture of 15 C23O subfamily I.2.A gene variants. Lanes 19 and 20 show AFDRA of the PCR amplification product obtained from 10^{-5} or 10^{-7} ng of an equimolar mixture of 14 C23O gene variants supplemented with a 10-fold excess of the *P. putida* CF600 gene. Lanes 21 and 22 show AFDRA of the PCR amplification product obtained from 200 pg or 20 pg of soil DNA of the capillary fringe zone, lanes 23 and 24 AFDRA of the PCR amplification product obtained from 200 pg or 20 pg of soil DNA of the saturated zone, lanes 25 to 28 AFDRA of the C23O genes from *P. stutzeri* AN10, *P. putida* CF600, *P. putida* G7, and *P. putida* mt-2, respectively. Lanes M were loaded with 120 ng of DNA Molecular Weight Marker V (Roche).

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CHAPTER III

Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries[§]

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Keywords

PCR-SSCP molecular fingerprint, BTEX contamination, catechol 2,3-dioxygenase, gene diversity, catabolic potential, biodegradation, natural microbial communities.

FUNCTIONAL GENE DIVERSITY ANALYSIS IN BTEX CONTAMINATED SOILS BY MEANS OF PCR-SSCP DNA FINGERPRINTING: COMPARATIVE DIVERSITY ASSESSMENT AGAINST BACTERIAL ISOLATES AND PCR-DNA CLONE LIBRARIES

3.1 Summary

Developments in molecular biology based techniques have led to rapid and reliable tools to characterize microbial community structures and to monitor their dynamics under in-situ conditions. However, there has been a distinct lack of emphasis on monitoring the functional diversity in the environment. Genes encoding catechol 2,3-dioxygenases (C23O), as key enzymes of various aerobic aromatic degradation pathways, were used as functional targets to assess the catabolic gene diversity in differentially BTEX contaminated environments by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP). Site specific PCR-SSCP fingerprints were obtained, showing that gene diversity experienced shifts correlated to temporal changes and levels of contamination. PCR-SSCP enabled the recovery of predominant gene polymorphs, and results closely matched with the information retrieved from random sequencing of PCR-DNA clone libraries. A new method for isolating strains capable of growing on BTEX compounds was developed to diminish pre-selection or enrichment bias and to assess the function of predominant gene polymorphs. C23O abundance in isolates correlated with the levels of BTEX pollution in the soil samples analysed. Isolates harbouring C23O genes, identical to the gene polymorph predominant in all contaminated sites analysed, showed an unexpected benzene but not toluene mineralising phenotype whereas isolates harbouring a C23O gene variant differing by a single point mutation and observed in highly polluted sites only, were capable, among some other isolates, to mineralise benzene and toluene, indicating a catabolically determined sharing of carbon sources on-site. The PCR-SSCP technique is thus a powerful tool for assessing the diversity of functional genes and the identification of predominant gene polymorphs in environmental samples as a prerequisite to understand the functioning of microbial communities.

3.2 Introduction

There is a growing interest in understanding how microbial communities react to challenging conditions, e.g., how they adapt to increasing levels of pollution. Genetic techniques such as Polymerase Chain Reaction (PCR) amplification using universal primers targeting 16S rRNA genes, allow the cultivation-independent examination of microbial community structures and their responses under stressful conditions. Many previous studies have focused on the taxonomic description of natural microbial communities, their changes and dominant key players (Torsvik and Ovreas, 2002). However, various functional genes such as catabolic genes are often localized on mobile genetic elements susceptible to high mutation rates and horizontal transfer (Lawrence, 2002; Williams *et al.*, 2002). Therefore, a certain level of metabolic potential or activity is not necessarily reflected by the taxonomic affiliation of organisms or by the microbial community structure. PCR-based methods have been successfully applied for detecting catabolic genes in the environment and quantitative PCR methods been demonstrated to be powerful tools for monitoring bioremediation processes (Stapleton and Sayler, 1998; Widada *et al.*, 2002). Such methods, however, have focused mainly on the characterisation of the presence, or abundance of a family of catabolic genes (Baldwin *et al.*, 2003; Beller *et al.*, 2002; Marlowe *et al.*, 2002; Mesarch *et al.*, 2000; Meyer *et al.*, 1999; Sotsky *et al.*, 1994) without assessing the finer levels of sequence diversity. The diversity of catabolic gene sequences often reflects differences in substrate specificity or affinity (Beil *et al.*, 1998; Cerdan *et al.*, 1994; Parales *et al.*, 2000; Suenaga *et al.*, 2002), and single amino acid differences have been reported to drastically change substrate specificity of toluene dioxygenase (Beil *et al.*, 1998), regioselectivity of naphthalene dioxygenase (Parales *et al.*, 2000) and toluene 2-monooxygenase (Pikus *et al.*, 1997), or activity of muconate cycloisomerase (Kaulmann *et al.*, 2001). A more detailed picture of the catabolic gene structure and sequence diversity in environmental samples will, thus, significantly increase our knowledge of the functional potential of microbial communities. Moreover, shifts in catabolic gene structure will allow the deduction of the evolutionary fitness of catabolic genes, operons and their respective hosts. A variety of molecular fingerprinting approaches previously developed to assess community structure, via the analysis of 16S rDNA or rRNA diversity, may be applied to define functional gene structure (Widada *et al.*, 2002).

Benzene, toluene, ethylbenzene and xylenes, commonly referred to as BTEX, are important monoaromatic, environmental contaminants world-wide. Their aerobic degradation can proceed by various different pathways, involving either an activation of the aromatic ring by dioxygenation or monooxygenation reactions, or through a processing of the side chain(s) (Williams and Sayers, 1994). Whereas a relatively broad diversity of activation mechanisms is possible, these pathways usually converge in the formation of (substituted) catechols as central intermediates (Reineke, 1998) which, in the majority of already described pathways, are subject to extradiol cleavage. Extradiol dioxygenases are, thus, key enzymes in the degradation of aromatic compounds and many of such proteins and their coding sequences have been described, purified and characterised in the last decades. Examination of their evolutionary relationships (Eltis and Bolin, 1996) showed that the majority of extradiol dioxygenases with preferences for monocyclic substrates were, at the protein level, phylogenetically closely related, constituting the so-called family of I.2 extradiol dioxygenases. Subfamily I.2.A seems to be of particular importance for the degradation of monocyclic aromatic compounds (Eltis and Bolin, 1996). Members of this enzyme subfamily were reported to differ significantly in their substrate specificity (Kitayama *et al.*, 1996) and, thus, the metabolic network in environmental communities will be significantly interconnected with the C23O diversity. Due to their central role in aromatic catabolism, the catechol 2,3-dioxygenases (C23O) subfamily I.2.A genes have been analysed in studies of diverse environments and their presence has been observed in various contaminated sites (Cavalca *et al.*, 2000; Chatfield and Williams, 1986; Daly *et al.*, 1997; Mesarch *et al.*, 2000; Meyer *et al.*, 1999; Okuta *et al.*, 1998; Wikstrom *et al.*, 1996) however, no fingerprinting methods to generate reliable and characteristic profiles from different environmental samples and to effectively extract predominant or specific gene sequences have been reported. In this study we introduce the application of a modified polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis with the single-strand removal method previously adapted for molecular fingerprinting of microbial community structures (Schwieger and Tebbe, 1998), for determining the characteristic sequence types of C23O genes as an indicator of catabolic potential. These PCR-SSCP results are assessed by comparing sequence data from PCR-DNA clone libraries and C23O sequences and metabolic performance of microorganisms exhibiting C23O activity. PCR-SSCP is demonstrated to be a reliable and rational

tool to rapidly determine sequence diversity within a catabolic gene family in environmental samples.

3.3 Results and Discussion

3.3.1 Optimisation of PCR-SSCP for generation of C23O fingerprints

In order to analyse the sequence diversity of C23O genes in soils differing in contamination level, the PCR-SSCP technique was optimised to generate fingerprints of C23O genes from total DNA extracts. Even though (Fig. 1A) it was possible to resolve, e.g., the different 238 nucleotide C23O gene single-strand conformations of *P. putida* mt-2 and *P. putida* HS1 fragments, which share a DNA sequence similarity of 95%, PCR-SSCP resolution of single-strands from 13 reference strain PCR-amplifications was low, and higher concentrations (>300 ng) of single-strands resulted in multiple conformations of the same sequence (Fig. 1A and supplementary online material 3), a phenomenon previously reported for single-strand DNA molecules (Atha *et al.*, 2001; Schmalenberger *et al.*, 2001). When the PCR primer set to amplify this 238-bp fragment was applied to soil DNA, amplification products were obtained from only 3 out of 5 contaminated soil samples (A1Y, A3Y and 1Y). ssDNA of the PCR amplicons obtained from these soil samples did not generate clear banding patterns, indicating the presence of overlapping polymorphic sequences with similar electrophoretic mobilities, as observed in ssDNA artificial polymorphic mixtures of this fragment (supplementary online material 3).

An improved resolution was achieved using ssDNA obtained from 583-bp C23O fragments (Fig. 1B), allowing a discrimination of the main polymorphic ssDNA mobility of each different reference ssDNA. However, multiple conformations of the same sequence types were still observed, and trials to amplify the 583-bp from soil samples were successful only in the same 3 contaminated samples as it was observed for the PCR-amplification of the 238-bp fragment (1Y, A1Y and 3Y). In contrast, PCR amplifications of the 527-bp C23O fragments generated positive signals from all 5 contaminated samples, and even a slight positive signal from the non-contaminated soil. Thus, the PCR primers developed in the course of this study were more effective than the primers previously used to amplify C23O fragments from the environmental samples tested.

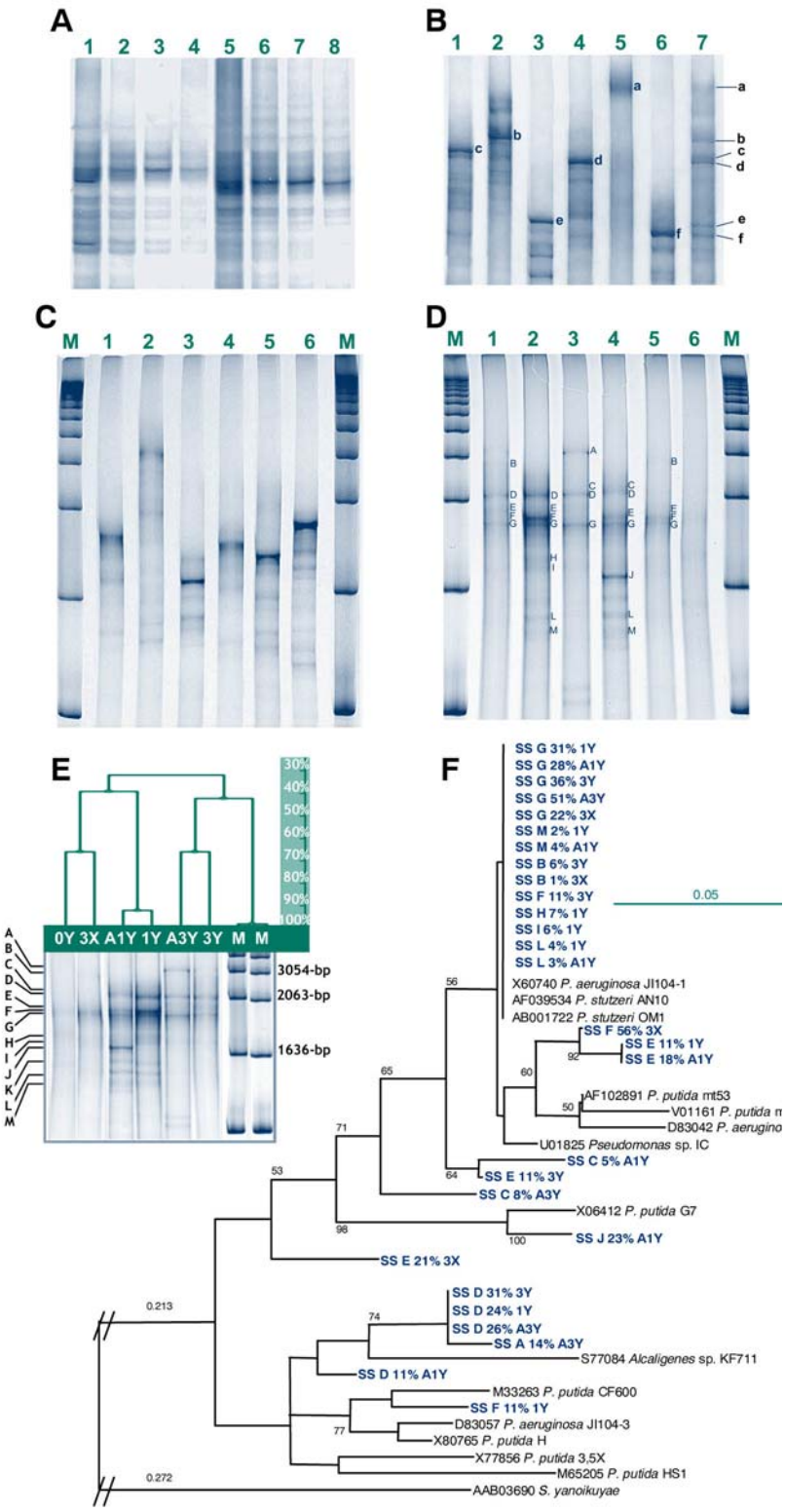

Fig. 1. Analysis of C23O gene fragments from reference strains or environmental DNA by PCR-SSCP with the single-strand removal method.
A. Mobility of 238b C23O ssDNA fragments amplified from *P. putida* mt-2 (lane 1-4) or *P. putida* HS1 (lane 5-8). A total of 520 ng (lane 1), 208 ng (lane 2), 104 ng (lane 3-4), 600 ng (lane 5), 240 ng (lane 6) or 120 ng (lane 7-8) was digested by lambda exonuclease for 3 h (lane 1-3 and 5-7) or 16 h (lane 4 and 8). B. Mobility of 583b C23O ssDNA fragments amplified from *P. stutzeri* AN10 (lane 1), *P. putida* CF600 (lane 2), *P. putida* G7 (lane 3), *Pseudomonas* sp. IC (lane 4), *P. putida* HS1 (lane 5), *P. putida* 3,5X (lane 6), and of a mixture of those amplification products (lane 7). The respective dominat bands are indicated by small letters. C. Mobility of 527b C23O ssDNA fragments amplified from *P. stutzeri* AN10 (lane 1), *P. putida* CF600 (lane 2), *P. putida* G7 (lane 3), *Pseudomonas* sp. IC (lane 4), *P. putida* HS1 (lane 5) and *P. putida* 3,5X (lane 6). M, 75 ng DNA Molecular Weight Marker X (Roche). D. Mobility of 527b C23O ssDNA fragments amplified from soil DNA, that has been extracted from samples 3Y (lane 1), 1Y (lane 2), A3Y (lane 3), A1Y (lane 4), 3X (lane 5) and 0Y (lane 6), respectively. Approximately 400 ng of dsDNA PCR product were digested and loaded. M, 75 ng of DNA Molecular Weight Marker X (Roche). Bands (A to M) subjected to sequence analysis are indicated by small letters. E. Dendrogram showing the degree of similarity of C23O gene structures as determined in D of samples 3Y, 1Y, A3Y, A1Y, 3X and 0Y (Dice coefficient, 5% confidence). Scale representing percentage of similarity between the profiles is shown at the left side. F. Phylogenetic tree of deduced C23O protein sequences from sequences recovered of PCR-SSCP bands, indicated as (SS), and designated according to sample origin and mobility (alignment of 99 amino acids, bar represents 5 amino acid changes per 100 amino acids). The sequences obtained were aligned with known members of the C23O subfamily I.2.A (indicated by their DDBJ/EMBL/GenBank accession number, organism of origin and strain designation). The C23O protein sequence of *S. yanoikuyae* B1, a member of the subfamily I.2.B, was used as an outgroup. Alignments were performed with CLUSTAL W using default values. N-J phylogenetic trees were generated using the option available in CLUSTAL W program. Bootstrap values above 50% from 1000 neighbor-joining trees are indicated to the left of the nodes.

PCR-SSCP analysis of the 527-bp C23O fragments showed clearly different mobilities of highly similar C23O fragments and one predominant single-strand conformation per reference strain (Fig. 1C). Fragment mixtures from environmental samples (Fig. 1D) produced strands of clearly differential mobilities, demonstrating the potential of the PCR-SSCP technique to retrieve polymorphic patterns of functional genes from the environment.

3.3.2 Diversity of C23O fingerprints and genetic information recovered from PCR-SSCP gels

To identify the predominant products by DNA sequencing, different C23O gene single-strands ("bands"), designated according to their electrophoretic mobilities as A – M (Fig. 1D) and representing at least 85% of the total signal intensity of each profile, were extracted from the gels and reamplified by PCR. All predominant bands observed in the profiles were composed of unambiguous single-strand species. This contrasts recent literature on profiling the 16S rDNA diversity by temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), or single-strand conformation polymorphism (SSCP), which showed that single bands may contain more than one sequence (Bruns *et al.*, 2001; Kisand and Wikner, 2003; Schmalenberger and Tebbe, 2003), a fact of specific importance if samples of high diversity are profiled. The profiling of a defined catabolic gene family with limited diversity, obviously lower the possibility of different single-strands having identical mobility. However, sequencing of low intensity bands showed some ambiguous bases in the sequence. Such ambiguous positions could be due to overlapping ssDNA with few sequence differences and present at lower concentrations, as reported in PCR-SSCP 16S rDNA studies (Schmalenberger and Tebbe, 2003). For this reason, only an unambiguous 301-bp block of the sequences recovered from the ssDNA bands, corresponding to nucleotides 314 to 614 of *P. putida* mt-2 C23O gene (protein positions 106 to 204) was used for phylogenetic analyses (Fig. 1E-F). The majority of bands with high relative intensities and identical mobility, like band G, present in all profiles, contained identical single-strands. In a few cases (e.g. bands G, M, H, I and L in profile 1Y), identical 301-bp sequences were recovered from different bands. Most probably, these different single-strand mobilities are due to sequence differences actually found in regions outside of the 301 base block analysed. This hypothesis is supported by cloned PCR-amplified C23O gene sequences wherein single base differences between closely related sequences were observed in these regions (see

below). The treatment of bands of identical sequence and mobility in different profiles as the same unique character, of identical sequences but different mobilities as different characters, and the treatment of different sequences exhibiting identical mobility as unique and independent characters, allowed the calculation of a dendogram showing the similarities between the PCR-SSCP profiles (Fig. 1E). High similarities were observed between PCR-SSCP profiles of samples from identical sites and horizons sampled at different time points, whereas low PCR-SSCP profile similarities were evident from samples differing in contamination level or taken from different soil horizons. These observations indicated that the catabolic gene composition might depend on the environmental conditions and is probably driven by the contamination level.

The sequence of band G, predominant in fingerprints of all contaminated samples is closely related to the *P. stutzeri* AN10 C23O gene sequence (Fig. 1F). Sequences closely related to the *Alcaligenes* sp. KF711 C23O gene were observed frequently in DNA extracted from capillary fringe soil samples. The sequence of a very intense and distinct ssDNA band, specific for the highly contaminated site A1Y, clustered within the isolated branch of the C23O gene of *P. putida* G7. Additionally, differences between highly-contaminated and low-contaminated sites were detectable. A sequence type identical to that retrieved from band E (97% similarity to *P. aeruginosa* JI104-1 C23O putative protein) was observed in highly contaminated samples only, whereas band A (closest relative *P. aeruginosa* ZD 4-3 C23O gene) was observed only in slightly contaminated samples. Thus, PCR-SSCP targeting the C23O gene was used successfully to identify subsets of C23O genes which are predominant throughout the whole contaminated area analysed, as well as subsets of genes which are predominant only at specific sites differing in contamination level or soil horizon.

3.3.3 Diversity of C23O sequences recovered from PCR clone libraries

The same 527-bp C23O gene PCR amplification products, from 1Y and 3Y total soil DNA, used to produce PCR-SSCP profiles were also used to generate clone libraries for determination of the diversity of C23O gene sequence types, a commonly used technique to assess diversity in a mixed PCR product (Hamelin *et al.*, 2002; Norton *et al.*, 2002; Yeates *et al.*, 2000). From the inserts, an unambiguous sequence length of 515-bp (corresponding to positions 193 to 707 or amino acids 65 to 235 of the *P. putida* mt-2 C23O gene) could be determined for each selected clone. The results (Fig. 2A) were in close agreement with the

information recovered from PCR-SSCP. A major clustering of sequence types closely related with *P. stutzeri* AN10 C23O – *P. aeruginosa* JI104 C23O was observed (Fig. 2A). The 18 distinct DNA sequences converge to only 5 putative protein sequence types indicating that the amino acid sequence integrity of the enzyme seems to be very important.

3.3.4 Isolation and characterisation of strains exhibiting C23O activity

The fact that C23O genes related to *Pseudomonas stutzeri* AN10 were observed to be dominant in all contaminated sites analysed raised the question on their importance for BTEX degradation. We thus aimed to isolate from the samples a broad collection of strains harbouring C23O activity. Analysis of reference strains revealed, that low levels of constitutive expression of C23O on R2A agar plates was sufficient to observe transformation of catechol into the yellow coloured 2-hydroxymuconic semialdehyde. Dilutions of soil samples were spread on R2A agar plates and all colonies were analysed for C23O activity by spraying with catechol. An extensive collection of strains exhibiting C23O activity could be isolated. Whereas there was no significant difference in the number of colony forming units from the differently contaminated soils tested (approximately 5 ± 3 x 10^6 CFU/g of soil), the percentages of colonies exhibiting *meta*-cleavage activity varied significantly, ranging from <3%, 10% and >70% of total CFU/g growing on R2A plates obtained from 0Y, 3Y and 1Y soil samples, respectively, correlating with the contamination levels. The environmental conditions and, probably, the contamination level were selective for strains possessing C23O activity. As total CFUs did not differ drastically it can be suggested that nutrients other than available carbon are limiting in the environment analysed. However, as the fraction of bacteria cultured here comprises only a subset of the total microflora, such assumptions remain speculative.

A high proportion (37 of 46 isolates) of the strains expressing *meta*-cleavage activity could use at least one of the BTEX compounds as a sole carbon source (Table 1), showing a high correlation between those characteristics. The DNAs of the isolates were used as templates in order to amplify C23O subfamily I.2.A gene fragments. Primer sets amplifying 238-bp, 527-bp and 583-bp fragments all showed successful amplification in the majority (26 out of 37) of isolates capable to grow on BTEX compounds. Some of the isolates exhibiting *meta*-cleavage activity and the ability to grow on BTEX compounds (11 isolates) were not amplifiable with C23O specific primers. As, besides members of C23O subfamily I.2.A, enzymes of

subfamily I.2.C and I.3.B also have been shown to be involved in the degradation of BTEX (Eltis and Bolin, 1996) it is highly probable, that this minor fraction of isolates contain genes of these subfamilies. A high proportion of 14 isolates, 13 of them carrying a C23O subfamily I.2.A gene, was capable, under the conditions tested, to grow only on benzene as a sole source of carbon, and microorganisms with such a phenotype have rarely been reported.

A selection of 30 isolates, preferentially those showing catabolic capabilities against BTEX or an amplifiable C23O fragment, were analysed by ARDRA, and three distinct *Alu*I ARDRA profiles were observed (Fig. 2B and Table 1). 16S rDNA sequencing of 13 representative strains revealed highest similarities to ribosomal sequences of *Pseudomonas* spp.: 6 strains exhibiting ARDRA profile PS1, 99.0 % to *P. fluorescens* DSM 50108, 6 strains exhibiting ARDRA profile PS2, 98.4 % to *P. brassicacearum* str. NFM421, and 1 strain exhibiting ARDRA profile PS3, 98.2 % to *P. corrugata* ATCC 17484.

3.3.5 Diversity of C23O genes from isolates

A primer pair annealing at the start and the stop codons of C23O I.2.A genes was designed and it showed amplification efficiency similar to that of primer pairs amplifying gene fragments. Use of this primer pair allowed not only to determine the whole gene sequence but a very convenient direct cloning and overexpression of C23O genes from isolates. The estimated phylogeny of the proteins deduced from PCR-amplified C23O gene sequences (Fig. 2C) from 19 isolates, comprising different morphotypes and BTEX degradation profiles, showed five distinct groups of sequence types. The predominant C23O DNA sequence type observed, identical in 11 different isolates (Fig. 2C), designated A1, differed from the *P. stutzeri* AN10 sequence (Bosch *et al.*, 2000) by only a single nucleotide, which represents one amino acid difference in position 218 (*Tyr* instead of *His*) and from the *P. aeruginosa* JI104-1 C23O sequence by six nucleotides, corresponding to only two amino acid differences in the putative protein sequence (*Ala* instead of *Arg* at position 60, and *Tyr* instead of *His* at position 218). This sequence type was identical to sequence recovered from the most intense bands of the SSCP profiles (bands G, Fig. 1F), and to the most abundant sequence retrieved from the C23O PCR clone libraries (Fig. 2A, group 1). A sequence identical to *P. stutzeri* AN10 C23O, designated type A2 (Fig. 2C) was observed in two isolates from site 1Y only as well as in two clones from the clone library of sample 1Y (Fig. 2A, group 5). The sequence difference between a type A1 and a type A2 sequence is not included in

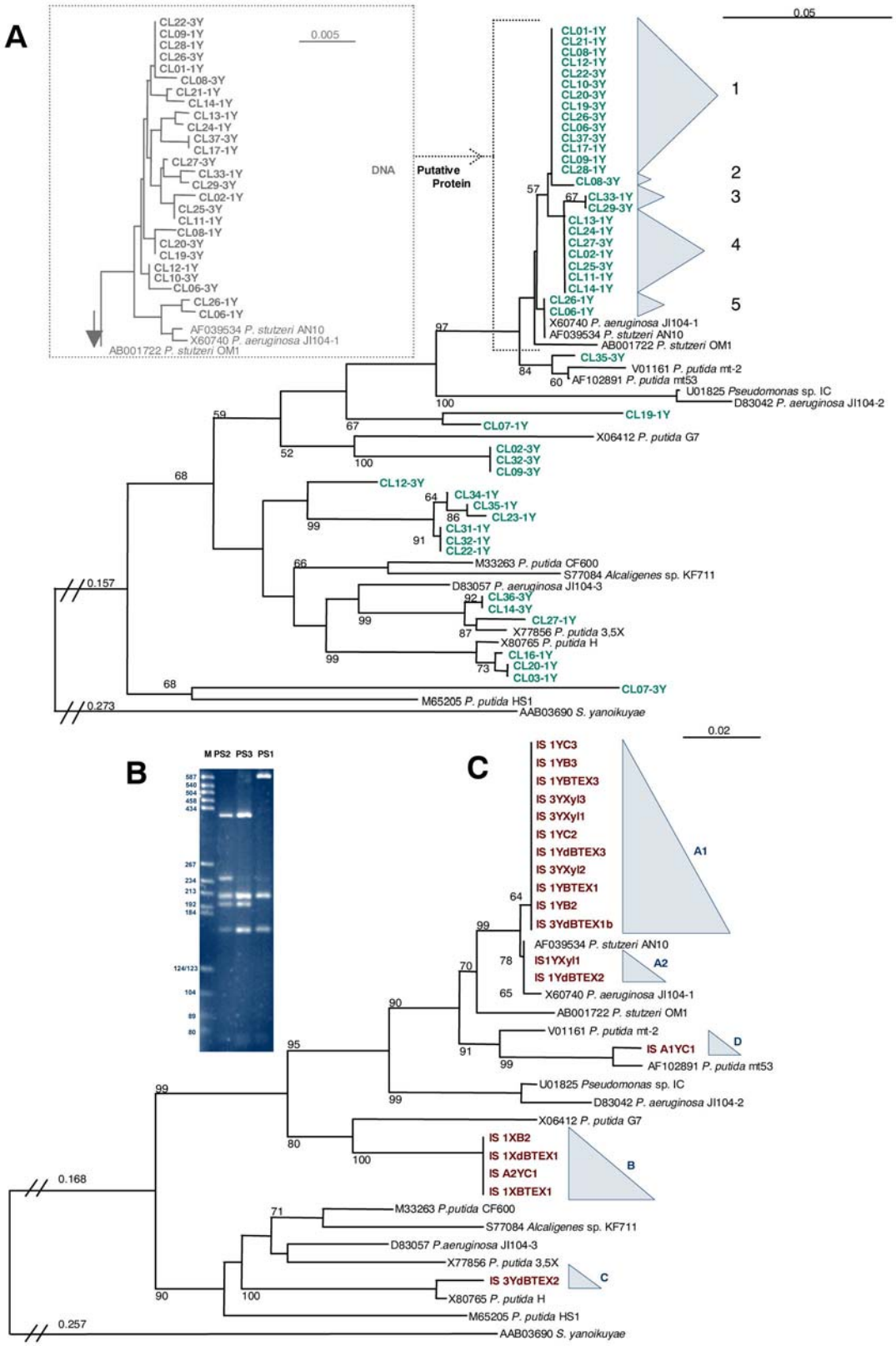

Fig. 2. Phylogenetic trees of deduced amino acid sequences of C23O gene fragments from PCR clone libraries and isolates and characteristic ARDRA profiles of isolates. Sequences were aligned with known members of the C23O subfamily I.2.A (indicated by their DDBJ/EMBL/GenBank accession number, organism of origin and strain designation). The C23O protein sequence of *S. yanoikuyae* B1, a member of the Subfamily I.2.B, was used as an outgroup. Alignments were performed with CLUSTAL W using default values. Phylogenetic trees were generated using the option available in CLUSTAL W program. Bootstrap values above 50% from 1000 neighbor-joining trees are indicated to the left of the nodes. **A**. Phylogenetic tree of deduced C23O protein sequences from PCR clone library sequences, indicated as (CL) and designated according to the site of origin (alignment of 171 amino acids, bar represents 5 amino acid changes per 100 amino acids). The insert shows the phylogenetic tree from the DNA alignment of PCR clone sequences closely related to *P. stutzeri* AN10 C23O (alignment of 513 nucleotides, bar represents 5 nucleotide changes per 1000 nucleotides). Triangles represent the 5 groups of identical putative protein sequences originating from 18 different DNA sequences. **B**. ARDRA types of isolates. **C**. Phylogenetic tree of deduced C23O protein sequences from bacterial isolates, indicated as (IS), from sites 3Y, 1Y, and 1X. (alignment of 268 amino acids, bar represents 2 amino acid changes per 100 amino acids). Triangles at the left side indicate groups of sequences (A to D) found in the strain collection.

the 301 base block used for phylogenetic analysis of PCR-SSCP bands. A detailed sequence analysis upstream of this block, however, gave no indications for the massive presence of an A2-type sequence in any of the analysed bands. A second C23O sequence type, retrieved from 4 isolates, was related to the isolated branch of the *P. putida* G7 C23O gene in the I.2.A subfamily. This sequence type could also be detected by PCR-SSCP or in the clone library (Fig. 1F and 2A) Two isolates contained C23O sequences, closely related to the *P. putida* mt53 (only found by this method) or the *P. putida* H C23O gene, respectively (also found in 3 of 27 clones from 3Y PCR library). The general phylogenetic distribution of the sequences from the isolates showed a clear predominant sequence polymorphism in organisms from soil samples 1Y and 3Y, as well as the coexistence of C23O genes of more distant evolutionary origins in isolates recovered from the same environment.

10 of 11 isolates that harboured a C23O type A1 use only benzene as a sole carbon source,-whereas both isolates harbouring a C23O type A2, were capable to grow on benzene, toluene and ethylbenzene. Isolate A1YC1 (C23O type D) can use only toluene, *m*-xylene and *p*-xylene as a sole carbon source. Its C23O sequence is closely related to xylE genes of TOL plasmids pWW0 and pWW53, and the catabolic phenotype of the isolate hints on the degradation of toluene and xylenes via the respective benzoates and thus a localization of the gene in a operon similar to the classical TOL pathway. Whereas there is a strong correlation between the degradative phenotype and the C23O type observed, there is no such correlation between the exact phylogenetic position of the isolates and the C23O gene variant they contain. As an example, strains 1YdBTEX2 and 1YB3 had exactly the same 16S rDNA sequence, but differed in growth phenotype and C23O gene comprised. On the other hand, 1YdBTEX2 and 3YdBTEX1 harboured the same C23O gene and shared the same growth phenotype, but differed in their 16S rDNA sequences and probably belong to different Pseudomonas species.

As there is a strong relationship between type of C23O present and growth phenotype, the question should be answered if, and in which way this gene and gene product or the operon it is located in, influences the growth phenotype. It could be reasoned that observation of C23O is just fortuitous and that growth on benzene only is due to an induction of a catechol *ortho*-cleavage route, a pathway reported not to be suited for the degradation of methylaromatics including toluene (Knackmuss *et al.*, 1976). However, control experiments using five isolates with different growth phenotype and C23O genes (1YC2 and 1YB2, type A1; 1Yxyl1,

1YdBTEX2, type A2; 1XB2, type B) showed that all those isolates exhibited high C23O activity (1000 – 4000 U/g protein), but negligible catechol 1,2-dioxygenase activity (< 50 U/g protein) during growth on benzene and thus use a *meta*-cleavage pathway. Two isolates, 1YB2 and 1YdBTEX2, representing type A1 and type A2 hosts, were selected for further analysis. Both strains showed a high constitutive expression of catechol 2,3-dioxygenase of 650 and 2900 U/g protein, respectively, only slightly lower than the activity during growth of benzene (Table 2). The C23O genes of both strains were overexpressed in *E.coli* and the gene products compared to those expressed during growth on glucose or benzene. Despite the difference in only one amino-acid residue, the substrate specificities of the overexpressed type A1 and type A2 enzymes differed significantly (Table 2). Whereas the type A2 *His*218 variant of 1YdBTEX2 showed, among the substrates tested, the highest activity with catechol, the type A1 *Tyr*218 variant of 1YB2 exhibited highest activity with 4-methylcatechol and a relatively elevated activity with 3-methylcatechol. An influence of this amino acid on substrate specificity was not expected from the reported crystal structure of the C23O of *P. putida* mt-2 (Kita *et al.*, 1999).

Partially purified enzymes from glucose grown cells of either 1YB2 or 1YdBTEX2 exhibited substrate specificities identical to the respective overexpressed proteins and showed identical retention behaviour during anion exchange chromatography (elution at 0.34 M NaCl). Similarly, only one C23O activity was observed when analysing proteins of benzene grown cells of either 1YB2 or 1YdBTEX2 by anion exchange chromatography.

To further evidence the involvement of the respective type A1 or type A2 C23O in degradation of benzene, aliquots of highly active fractions obtained by anion exchange chromatography were subjected to SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and major protein bands of a molecular mass of 35 ± 2 Kda analysed by N-terminal sequencing. The amino-terminal sequences of the proteins from 1YB2, 1YdBTEX2, JM109(pA1Tyr) and JM109(pA2His) were identical and identified as MNKGIMRPGHVQL; this sequence is identical to the sequence of the C23O proteins of *P. stutzeri* AN10 and *P. aeruginosa* JI104. Inner sequences WGVNEVNPEAWPR and FQAPSGHHFELYADKEYTGK obtained from both the 1YB2 and 1YdBTEX2 derived proteins were identical to those deduced from the type A1 and type A2 C23O gene sequences. Thus, the C23O activity constitutively expressed in glucose-grown cells is due to a type A1 C23O in 1YB2 and to a type A2 C23O in 1YdBTEX2.

Table 1

Utilization of single BTEX compounds as sole carbon sources by bacterial isolates exhibiting *meta*-cleavage activity.

Isolate Name	B	T	E	m-X	p-X	o-X	C23O	ARDRA type
A1YC1	-	+	-	+	+	-	+	PS2*
A1YC2	-	+	-	+	+	-	+	PS2*
1Y C1	+	+	+	-	-	-	+	PS1
1Y C2	+	-	-	-	-	-	+	PS1*
1Y C3	+	-	-	-	-	-	+	PS1
1Y Xyl1	+	+	+	-	-	-	+	PS1
1Y Xyl2	+	+	+	-	-	-	+	PS1
1Y Xyl3	+	+	+	-	-	-	+	PS1
1Y dBTEX1	-	-	-	-	-	-	+	ND
1Y dBTEX2	+	+	+	-	-	-	+	PS1*
1Y dBTEX3	+	-	-	-	-	-	+	PS1
1Y BTEX1	+	-	-	-	-	-	+	PS1
1Y BTEX3	+	-	-	-	-	-	+	PS1
1Y B1	+	-	-	-	-	-	+	PS1
1Y B2	+	-	-	-	-	-	+	PS1*
1Y B3	+	-	-	-	-	-	+	PS1*
3Y C1b	+	+	-	-	-	-	-	ND
3Y C1	-	-	-	-	-	-	-	ND
3Y C2	+	+	+	-	-	-	-	ND
3Y C3	+	-	-	-	-	-	-	ND
3Y C4	+	+	+	-	-	-	-	ND
3Y C5	+	+	-	-	-	-	+	PS2
3Y Xyl2	+	+	-	-	-	-	-	PS2*
3Y Xyl1	+	+	-	-	-	-	+	PS2
3Y Xyl2b	+	-	-	-	-	-	+	PS2
3Y Xyl3	+	-	-	-	-	-	+	PS2
3Y dBTEX1	-	-	-	-	-	-	-	PS2*
3Y dBTEX2	+	+	+	-	-	-	+	ND
3Y dBTEX1b	+	-	-	-	-	-	+	PS2*
3Y BTEX2b	+	+	+	-	-	-	-	PS2*
3Y B1	-	-	-	-	-	-	-	ND
3Y B1b	-	-	-	-	-	-	-	ND
3Y B2	-	-	-	-	-	-	-	ND
1X C1	+	+	+	-	-	-	-	ND
1X C2	+	+	-	-	-	-	+	PS2
1X Xyl1	+	+	+	-	-	-	-	ND
1X Xyl1b	+	-	-	-	-	-	-	ND
1X Xyl2	-	-	-	-	-	-	+	PS1
1X dBTEX1	-	-	-	-	-	-	+	PS1*
1X BTEX1	+	+	+	-	-	-	+	PS1
1X BTEX3	+	+	+	-	-	-	-	PS1
1X BTEX4	+	+	+	-	-	-	+	PS1
1X BTEX1b	+	+	+	-	-	-	-	ND
1X B1	+	-	-	-	-	-	+	PS3*
1X B1b	-	-	-	-	-	-	+	ND
1X B2	+	+	+	-	-	-	+	PS1*

Strains were designated according to sample origin and aromatic cosubstrates included during isolation on R2A plates prior as described in the experimental procedures. Growth was determined on solid mineral medium supplemented with single aromatic substrates as sole carbon and energy source. Aromatic substrate used were benzene (B), toluene (T), ethylbenzene (E), *meta*-xylene (m-X), *para*-xylene (p-X) and *ortho*-xylene (o-X). All isolates were analysed for the presence of C23O subfamily I.2.A amplifiable gene. (+), PCR product of the expected length by all 4 primers, (-) no PCR product by any of the primers. A selection of isolates were analysed by ARDRA and the respective banding profile (ARDRA type PS1, PS2 or PS3, respectively, Fig. 2B) is indicated. (*) denote those isolates further analysed by full sequencing of the 16S rDNA PCR fragment. ND, not determined.

Table 2

Substrate specificity and activity of catechol 2,3-dioxygenases of strains 1YB2, 1YdBTEX2, *E.coli* JM109 (pA1Tyr) and *E.coli* JM109 (pA2His).

	Strain			
Substrate	1YB2	1YdBTEX2	JM109(pA1Tyr)	JM109(pA2His)
Catechol	100 (830 ± 40)	100 (4.200 ± 1800)	100 (1.300 ± 80)	100 (4.800 ± 200)
3-Methylcatechol	45 ± 5	20 ± 5	45 ± 5	20 ± 5
4-Methylcatechol	120 ± 10	50 ± 10	140 ± 20	50 ± 10
4-Chlorocatechol	40 ± 5	35 ± 5	45 ± 10	35 ± 5

Activities were determined at substrate concentrations of 0.1 mM and are given relative to that with catechol (100%). Activities in cell extracts (U/g protein) are given in parentheses.

However, the observed differences in substrate specificity do not explain the difference in growth behaviour, as the type A1 C23O of benzene degraders is highly active with methylsubstituted catechols. It was therefore analysed if strain 1YB2 contains an active 2-hydroxymuconic semialdehyde hydrolase (xylF). XylF is necessary for degradation of toluene via 3-methylcatechol and 2-hydroxy-6-oxo-hepta-2,4-dieneoate, but dispensable for growth on benzene via catechol and 2-hydroxymuconic semialdehyde, as 2-hydroxymuconic semialdehyde can be degraded via the alternative branch of the meta-cleavage pathway involving a 2-hydroxymuconic semialdehyde dehydrogenase, xylG (Sala-Trepat *et al.*, 1972). Intriguingly 1YB2 as well as 1YdBTEX2 showed constitutive expression of high levels of a 2-hydroxymuconic semialdehyde hydrolase of 170 ± 20 U/g protein against 2-hydroxy-6-oxo-hepta-2,4-dieneoate and of 70 ± 10 U/g protein against 2-hydroxymuconic semialdehyde and of a 2-hydroxymuconic semialdehyde dehydrogenase (20 ± 5 U/g and 65 ± 10 U/g protein in cell extracts of 1YB2 or 1YdBTEX2, respectively). It is thus evident, that the difference in growth phenotype of 1YB2 and 1YdBTEX2 is not due to substrate specificity differences of C23O enzymes, nor to the absence of a functional 2-hydroxymuconic semialdehyde hydrolase.

Nevertheless, as the presence of a A1 type C23O gene is directly correlated with a benzene, and the presence of a A2 type C23O gene with a benzene/toluene degrading phenotype, it can be assumed that these C23O genes are linked, possibly in an operon, with other catabolic pathway genes, which either, by their substrate specificity or by controlling metabolic flux through the pathway, determine the range of substrates that can be mineralized. Specifically, the accumulation of catechols formed during metabolism is known to prevent growth of single organisms on aromatic compounds (Perez-Pantoja *et al.*, 2003).

3.4 Conclusions

The alignment of a common putative 99 amino acid stretch derived from C23O gene sequence information obtained from bacterial isolates, PCR clone libraries and extracted PCR-SSCP bands, allowed a comparison of the three different methods applied (Fig. 3), specifically for soil samples 1Y and 3Y. All the methods showed a predominance of sequences related to the *P. stutzeri* AN10 C23O gene. The diversity of C23O gene sequences from isolates was, as expected, lower. As an example, the second most commonly observed C23O gene sequence observed in the clone library and the PCR-SSCP profile, related to the *P. putida* CF600 C23O

gene, was not observed in any of the isolates. As 20 and 27 cloned PCR-amplified C23O genes were sequenced for sites 3Y and 1Y respectively, the sequence types detected at higher numbers can be assumed to be more prevalent in the samples. Those sequences were also predominant in the PCR-SSCP profiles. Thus, PCR-SSCP profiling has provided a reliable overview of the relative abundance and diversity of C23O genes within different environmental conditions. We have shown a successful example of adaptation of PCR-SSCP to generate fingerprints of functional genes, which makes very likely further developments, optimisations and applications of this technique to target a number of gene families playing functional roles in the environments. Together with promising methods to characterise functional gene expression in environmental samples (Koizumi *et al.*, 2002; Manefield *et al.*, 2002; Marlowe *et al.*, 2002; Miskin *et al.*, 1999; Wawer *et al.*, 1997), this technique would help future trends to characterize and compare catabolic gene diversity and structure, as well as active operons and pathways under changing environmental conditions.

Even though the expression of those genes upon BTEX stress has not been proven directly in the environmental samples, and could be constitutive as in the isolates, it appears evident that specific C23O genes and operons have been positively selected during the adaptation of soils to BTEX contamination. Moreover, from studies on isolates we could show that the presence of a C23O type A1 gene seems to be related to a benzene degrading phenotype. Those genes were dominant in all samples analysed and can be related to the fact that benzene was the major pollutant in all those samples. The isolates represent a new, not previously reported growth phenotype of obvious selective advantage under environmental conditions. Whereas organisms harbouring a C23O type A1 containing pathway are highly specialized, organisms harbouring C23O type A2 genes, from studies of isolates, can be related to a benzene/toluene degrading phenotype, and were detected only in highly contaminated samples, which had been stressed with a significant amount of toluene in addition to benzene. If such kind of pathway actually evolved from a pathway comprising a C23O type A1 remains to be elucidated. Nevertheless, the application of a multifaceted approach by a new PCR-SSCP of functional genes, its validation by clone libraries and a non-biased isolation of BTEX degraders and their biochemical characterization allowed a better

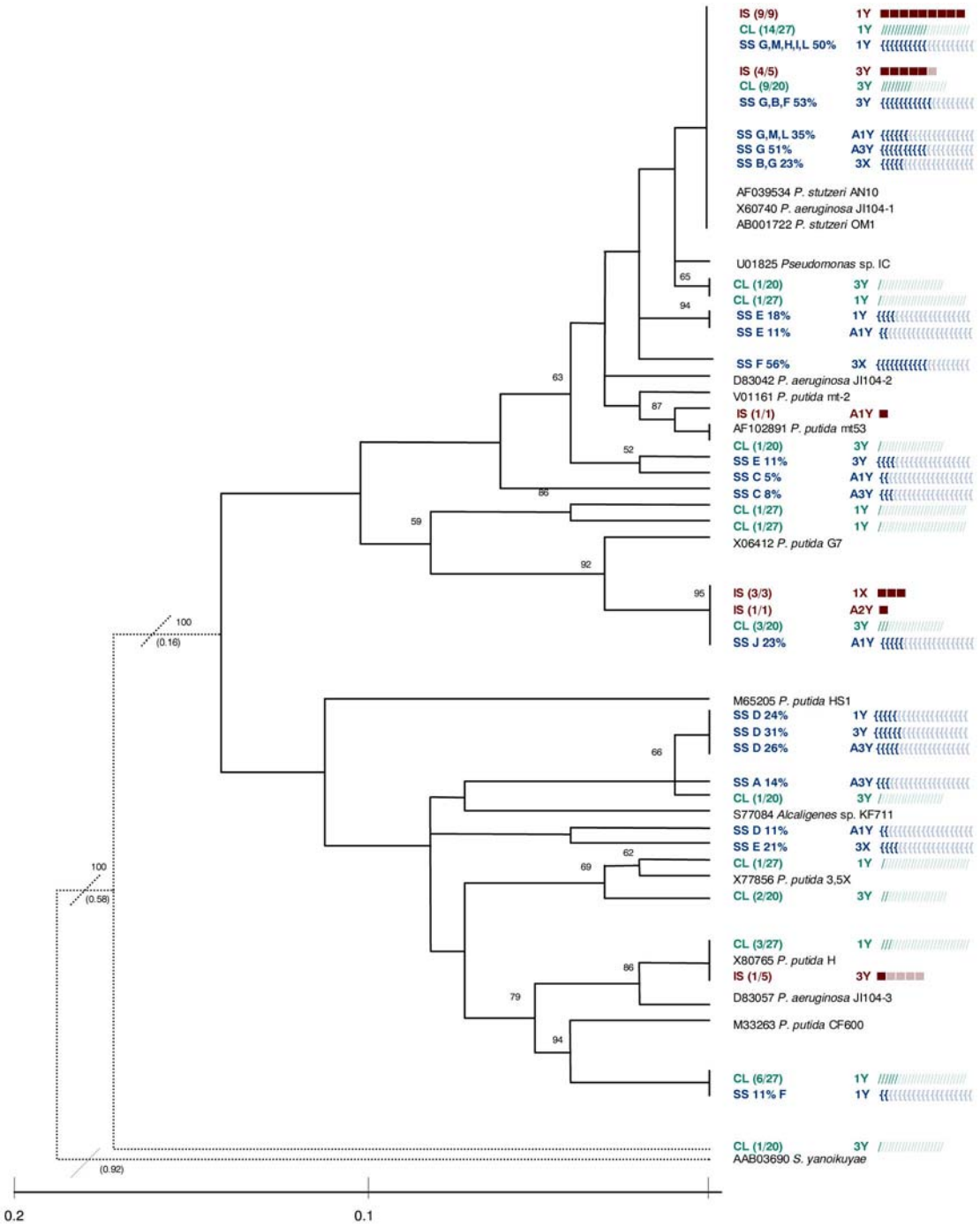

Fig. 3. Cumulative joint UPGMA clustering representation of related sequences from PCR-SSCP bands (SS), clone libraries (CL) and isolates (IS) (99 amino acids, scale represents similarity coefficient). The sequences obtained by all methods were aligned with known members of the C23O subfamily I.2.A (indicated by their DDBJ/EMBL/GenBank accession number, organism of origin and strain designation). The C23O protein sequence of *S. yanoikuyae* B1, a member of the subfamily I.2.B, was used as outgroup. The site of origin of the sequence retrieved is indicated at the right border together with a graphical representation of relative abundance. The abundance of a specific C23O sequence from isolates (IS) is given as number of isolates showing this sequence/number of total isolates from that site, and additionally by (■) indicating the number of isolates with the respective sequence, and (■) indicating the number of other isolates from that site. The abundance of C23O sequence from clone libraries (CL) is given as ratio of clones showing this sequence/number of total clones from that site, and additionally represented by (/) indicating the number of isolates showing this sequence, and (/) indicating the number of other clones from that site. The abundance of C23O sequence from PCR-SSCP bands (the respective mobilities are indicated) is given as percentage of total signal intensity, which is additionally represented by ({) in front of the sequence. Total of black and grey left curly braces correspond to 100%.

understanding on the complexity of BTEX degradation in the environment and gives a metabolic explanation to the fact that complex communities are involved in mineralisation. Currently microcosm experiments and on site studies are performed to analyse shifts in catabolic gene composition in more detail.

3.5 Experimental Procedures

3.5.1 Microorganisms, samples, isolation and culture conditions

Reference strains used in this study were *P. stutzeri* AN10 (Bosch *et al.*, 2000), *P. putida* CF600 (Bartilson and Shingler, 1989), *P. putida* G7 (Ghosal *et al.*, 1987), *P. putida* H (Herrmann *et al.*, 1995), *Pseudomonas sp.* IC (Carrington *et al.*, 1994), *P. aeruginosa* JI104 (Kitayama *et al.*, 1996), *Sphingomonas sp.* KF711 (Moon *et al.*, 1996), *P. putida* mt-2 (Nakai *et al.*, 1983), *P. putida* mt53 (Keil *et al.*, 1985), *P. stutzeri* OM1 (Ouchiyama *et al.*, 1998), *P. stutzeri* OX1 (Arenghi *et al.*, 2001), *P. putida* HS1 (Benjamin *et al.*, 1991) and *P. putida* 3,5X (Hopper and Kemp, 1980). Bacterial strains were cultured on R2A agar at 30°C for 3 days, and stored at –70°C in glycerol stocks (Sambrook *et al.*, 1989).

Soil samples were collected from the unsaturated (X) and the capillary fringe (Y) horizons from an aquifer located in the Czech Republic, which carries an underground BTEX plume. The sampling sites were designated 0 (non polluted soil), 3 (slightly BTEX contaminated soil), and 1 (highly BTEX contaminated soil). In two successive sampling campaigns 2001 - 2002, the BTEX concentrations in the groundwater were determined (Aquatest a.s. Prague) ranging from 320, 97, 6 and 13 mg/l of benzene, toluene, ethylbenzene and xylenes respectively (site 1), to 55, 2, 0.007 and 0.012 mg/l of benzene, toluene, ethylbenzene and xylenes respectively (site 3). Samples were designated according to sampling site (0, 1 or 3) and soil horizon (X or Y). To differentiate between samples times, samples collected in 2001 were additionally designated by an A.

Microorganisms with C23O activity were isolated by plating 0.1 ml of appropriate dilutions of soil samples onto R2A agar plates incubated in the absence or presence of aromatic compounds (benzene (B), *m*-xylene (Xyl), an equimolar mixture of BTEX (BTEX) or a 1:100 dilution of the equimolar mixture of BTEX (dBTEX) dissolved in heptamethylnonane). In each case, 20 µl of substrate were supplemented via the vapour phase by placing them into eppendorf pipette tips inside the petri dishes. After 3 days of incubation at 30°C, plates were screened for the presence of colonies exhibiting C23O activity by spraying with an aqueous

solution of catechol (100 mM). A subset of colonies with different colony morphotypes and exhibiting yellow coloration upon spraying were selected and purified. The utilization by the isolates of single BTEX compounds as a sole carbon an energy source was determined by growth on mineral medium plates (Dorn *et al.*, 1974) solidified with SeaKem LE Agarose (FMC Bioproducts) and supplemented via the vapour phase with 20µl of benzene, toluene, ethylbenzene or *o*-, *m*-, or *p*-xylene. To discard false positives, the growth results were assessed by comparison with the growth characteristics of the isolates on mineral medium agarose plates.

3.5.2 Primer design for amplification of C23O subfamily I.2.A gene fragments

In the literature, different primer sets, have been reported. Out of previously described primers pairs specific to C23O subfamily I.2.A genes (Mesarch *et al.*, 2000; Meyer *et al.*, 1999; Okuta *et al.*, 1998; Wikstrom *et al.*, 1996), a set targeting a 238-bp fragment, reported to be designed based on a multiple DNA sequence alignment of 8 reference was tested (Mesarch *et al.*, 2000). This primer pair amplified the expected PCR product size from all 13 reference strains, including *P. putida* G7 C23O (data not shown). Multiple DNA and protein alignments (supplementary online material 1) of 21 subfamily I.2.A C23O sequences were used to localize further conserved regions for primer design. Three new primer sets were found suitable to amplify the expected PCR product sizes of 527-bp, 583-bp and 934-bp from DNA of all 13 reference strains (example of 527-bp amplifications in supplementary online material 2). When DNA of isolates was used as template, similar positive PCR signals were obtained with each primer set (data not shown). Thus, the primer set producing the longest fragment of 934-bp was selected to amplify, sequence, and obtain recombinant expression of genes from the isolates.

3.5.3 DNA extraction and PCR

DNA from soil samples was extracted and purified with the Fast Prep Soil DNA Extraction Kit (BIO101). To extract DNA from bacterial strains, single colonies were boiled and centrifuged (Kanakaraj *et al.*, 1998), 4 µl of the supernatants were used as a PCR template in a final volume of 50 µl of a mixture containing 1X PCR Buffer (Promega) supplemented with 1.5 mM $MgCl_2$, 200 µM dNTP′s, 0.5 µM of each primer (synthesized by Invitrogen) and 0.3 U/µl Taq DNA Polymerase (Promega).
To amplify and sequence catechol 2,3-dioxygenase gene fragments the following primers were used: 23CAT-F 5' CGA CCT GAT CTC CAT GAC CGA 3' and DEG-R 5' TYA GGT CAK MAC GGT CA 3' to amplify a 238-bp C23O gene fragment (Mesarch

et al. 2000) comprising positions 687 to 924 of *P. putida* mt-2 xylE CDS, forward 5' ATG GAT TTY ATG GSB TTC AAG G 3' and reverse 5' TCG ATV GAK GTR TCG GTC ATG 3' to amplify a 527-bp C23O gene fragment corresponding to nucleotide positions 193 to 719 of *P. putida* mt-2 xylE gene (this study), forward 5' TCT AYC TSA AGG CYT GGA 3' and reverse 5' TCG GTC ATG GAG ATC AGG TCG 3' to amplify a 583-bp C23O gene fragment corresponding to nucleotide positions 125 to 707 of *P. putida* mt-2 xylE gene (this study), forward 5'AGG TGW CGT SAT GAA MAA AGG 3' and reverse 5' TYA GGT SAK MAC GGT CAK GAA 3' to amplify the complete C23O gene plus 10 bases upstream producing a fragment of an expected size of 934-bp, corresponding to nucleotide positions -10 to 924 of *P. putida* mt-2 xylE gene (this study). To determine optimal annealing temperatures for the primer sets, gradient PCR was performed with annealing temperatures between 50.4 to 60°C. All PCR programs comprised an initial step at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing for 45 s, and elongation at 72°C (the duration of the elongation step was varied according to the targeted PCR product size, using 10 sec per 100-bp) and a final elongation step at 72°C for 8 min. When the PCR products were intended to be applied on PCR-SSCP gels, annealing temperature of 52°C was applied. When the PCR products were amplified for direct sequencing, a touchdown PCR approach was used for 10 cycles, with an initial annealing temperature of 60°C followed by a decrease in annealing temperature of 1°C per cycle, followed by 25 PCR cycles at a constant annealing temperature of 55°C. To evaluate PCR product sizes, 4 µl of the PCR reactions were analysed by agarose gel electrophoresis (1.5% agarose, 10 cm length, 1X TAE running buffer, 1 hour at 95 volts). DNA bands were visualized by ethidium bromide staining (Sambrook *et al.*, 1989). To compare amplification efficiency among the primer sets, DNA extracted from *P. putida* mt-2 or a mixture of equal amounts of *P. putida* mt-2, *Pseudomonas* sp. IC, *P. putida* G7, and *P. aeruginosa* JI104 DNA were used as template, and serial dilutions were applied in PCR reactions with each primer set. Purification of PCR products was done with either the Qiaquick PCR Cleaning Kit or the Gel Extraction Kit (Qiagen) according to the manufacturer instructions. PCR amplification of 16S rDNA fragments from isolates was performed as previously described (Karlson *et al.*, 1993). 5 µl of the purified PCR products were subjected to amplified ribosomal DNA restriction analysis (ARDRA) using the restriction enzyme *Alu*I (Mocali *et al.*, 2003) and representatives of distinctive restriction patterns were fully sequenced.

The 934-bp C23O PCR fragments of 1YB2 (type A1) and 1YdBTEX2 (type A2) were cloned into pGEM-T by using the pGEM-T Easy Kit (Promega) to give pA1Tyr and pA2His, respectively. These plasmids were transformed into *Escherichia coli* JM109 competent cells (Promega). *E. coli* cells were cultured on LB medium at 37°C, supplemented with ampicillin at a final concentration of 100 µg/ml.

3.5.4 PCR-SSCP

Single-stranded DNA from PCR products was obtained as previously described (Schwieger and Tebbe, 1998). Briefly, PCR as performed with one of the primers being 5' phosphorylated, PCR products were eluted from agarose gels, and the phosphorylated strands were digested by lambda exonuclease (NEB). The remaining single-strands were purified with Qiaquick PCR Cleaning Kit (Qiagen), dried by vacuum centrifugation, resuspended in 6 µl of loading buffer (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol), and denatured for 5 minutes at 94 °C, followed by instant cooling on water ice bath for 3 minutes. The separation conditions were standardised in a DCode System for PCR-SSCP (Bio-Rad) coupled to a cooling bath device (Lauda E100).

Optimised running parameters were 120V (10 mA) for 18 h at a constant temperature of 26°C on 20 cm x 20 cm x 0,75 mm 0.6X MDE gels in 0.7X TBE (Sambrook *et al.*, 1989) as a running buffer. Optimal results were obtained when ssDNA obtained from 100 - 400 ng dsDNA was loaded onto the gels and a slightly enhanced resolution was achieved when the amplified single-strands of the reverse primer were subjected to PCR-SSCP analysis. For nucleic acid detection, gels were silver stained as reported previously (Bassam *et al.*, 1991). Single-strand electrophoretic mobilities corresponding to different conformations were excised from dried gels, and DNA extracted by the "Crush and Soak" method (Sambrook *et al.*, 1989). PCR reamplification of the excised and eluted single-strands was made with the same primers used to generate the original dsDNA fragment. PCR-SSCP bands of different mobilities were quantified by using Scion Image Beta 4.0.2 software (Scion Corporation) and Imagequant Software (Molecular Dynamics). The similarity matrix of PCR-SSCP fingerprints was calculated using Bio1D v.99.02 Software (Vilber Lourmat) either with Nei et Li (Dice) or Jaccard coefficients 5% confidence applied to both compared bands. A dendrogram of similarity values in the matrix was calculated using the UPGMA algorithm.

3.5.5 DNA sequencing and phylogenetic analyses

Nucleotide sequencing of PCR fragments or plasmids with cloned inserts was carried out on both strands using Taq dye-deoxy terminator in an ABI 373A automatic DNA sequencer (Perkin-Elmer Applied Biosystems) using M13 forward and reverse primers (Sambrook *et al.*, 1989). Primers used for sequence reactions were the same as those used in the original PCR. Contigs were assembled with Sequencher software (V 4.0.5). Alignments were generated with CLUSTAL X 1.8 windows interface of CLUSTAL W program using default values (Thompson *et al.*, 1997). Their results were edited and translated using GeneDoc (version 2.6.001). The Sequence Match program (Cole *et al.*, 2003) was used to find the closest relative to the 16S rDNA sequences obtained. Phylogenetic trees were obtained with the option available on CLUSTAL program through Neighbor-Joining (N-J) algorithm method. Distances were generated using the Kimura Matrix, and tree stability was supported through Bootstrap analysis, values higher than 50% of 1000 replications (seed value 111) are shown on respective nodes. Trees were visualized with NJplot (Perriere and Gouy, 1996). To visualize relationships between the sequences retrieved by all the methods, the program Treecon for Windows (1.3b) (Van de Peer and De Wachter, 1993) was used to estimate distances using a Kimura matrix with 500 bootstrap samples and to infer tree topology by UPGMA clustering method. Sequence data reported in this study have been submitted to the GenBank/EMBL//DBBJ databases and are available under accession numbers AJ544921 to AJ545012, AY228547, AY364085, AY364086 and AY364087.

3.5.6 Preparation of cell extracts

Isolates were routinely grown in mineral salts medium (Dorn *et al.*, 1974) containing 50 mM phosphate buffer (pH 7.4), supplemented with 4 mM of benzene (supplied via the vapour phase) or 1 % glucose. The baffled Erlenmeyer flasks were sealed with Teflon coated screw caps and incubated at 30°C on a rotary shaker (150 rpm). *E. coli* JM109 containing C23O genes were grown at 37°C in Luria broth medium containing 1 mM IPTG and 0.1 mg/ml ampicillin. Cells harvested from 100 – 200 ml of medium were resuspended in potassium phosphate buffer (50 mM, pH 7.5), and disrupted using a French press (Aminco, Silver Spring, MD, USA). Cell debris was removed by centrifugation at 100,000 x *g* for 1 h at 4^{o}C.

3.5.7 Enzyme assays

Catechol 2,3-dioxygenase activity was determined in 50 mM K/Na-phosphate (pH 7.5) with 0.1 mM of catechol, 3-methylcatechol, 4-methylcatechol or 4-chlorocatechol as substrates, using extinction coefficients of reaction products previously described (catechol, ε_{375nm} = 36,000 M^{-1} cm^{-1}; 3-methylcatechol, ε_{388nm} = 13,800 M^{-1} cm^{-1}; 4-methylcatechol, ε_{382nm} = 28,100 M^{-1} cm^{-1}; 4-chlorocatechol, ε_{379nm} = 39,600 M^{-1} cm^{-1}) (Heiss *et al.*, 1995; Hirose *et al.*, 1994). Catechol 1,2-dioxygenase was analyzed as previously described (Dorn and Knackmuss, 1978). Kinetic measurements were recorded on an UV 2100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). 2-Hydroxymuconic semialdehyde hydrolase was measured by determining the NAD-independent decrease in concentration of 2-hydroxymuconic semialdehyde (0.05 mM) at 375 nm or of 2-hydroxy-6-oxo-hepta-2,4-dieneoate (0.05 mM) at 388 nm (Sala-Trepat *et al.*, 1972). 2-Hydroxymuconic semialdehyde dehydrogenase was measured by determining the NAD-dependent decrease in concentration of 2-hydroxymuconic semialdehyd (0.05 mM) at 375 nm. NAD was added to a final concentration of 0.5 mM. 2-Hydroxymuconic semialdehyde dehydrogenase activity was calculated by subtracting the 2-hydroxymuconic semialdehyde hydrolase activity from the overall rate of 2-hydroxymuconic semialdehyde disappearance. 2-Hydroxymuconic semialdehyde and 2-hydroxy-6-oxo-hepta-2,4-dieneoate were prepared *in situ* by incubation of 0.05 mM solutions of catechol or 3-methylcatechol in phosphate buffer (50 mM, pH 7.4) with partially purified C23O from *E. coli* JM109 (pA2His) (corresponding to 0.1 – 0.2 U/ml). Protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

3.5.8 Enzyme purification and sequencing

Catechol 2,3-dioxygenases were partially purified using a Fast Protein Liquid Chromatography system (Amersham Biosciences, Freiburg, Germany). Cell extracts of *E. coli* JM109 (pA2His), *E. coli* JM109 (pA1Tyr), strain 1YB2 or strain 1YdBTEX2 were separately applied to a MonoQ HR 5/5 column and eluted by a linear gradient of NaCl (0 to 0.5 M over 25 ml) in Tris HCl (50 mM, pH 7.5) (flow rate 1 ml/min; fraction volume 0,5 ml). (SDS-PAGE) was performed on a Bio-Rad Miniprotein II essentially as described previously (Laemmli, 1970). The acrylamide concentrations for concentrating and separating gels were 5 and 10% (wt/vol), respectively. The proteins were stained by Coomassie brilliant blue R250. Proteins

were electroblotted onto a polyvinylidene difluoride membrane, and the membrane was stained with Coomassie brilliant blue R250. N-terminal amino acid sequencing was performed with an Applied Biosystems model 494A Procise HT sequencer. In-gel trypsin digests of proteins were analysed with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Reflex apparatus (Bruker) as previously described (Heim *et al.*, 2002). Respective peptide fingerprints obtained were compared by BLAST tool (Tatusova and Madden, 1999) against protein databases or putative proteins of gene or gene fragments sequenced in the course of this study.

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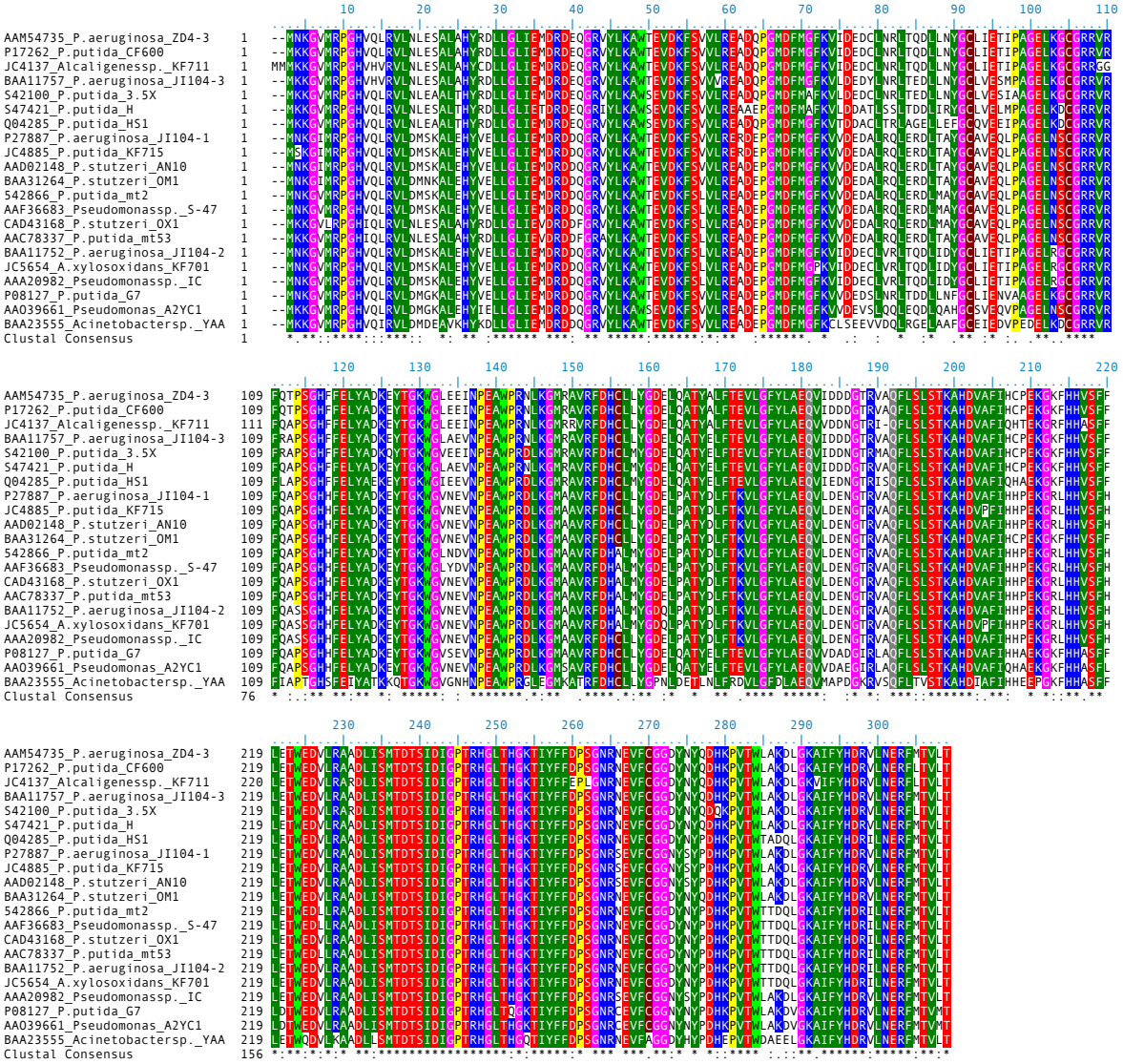
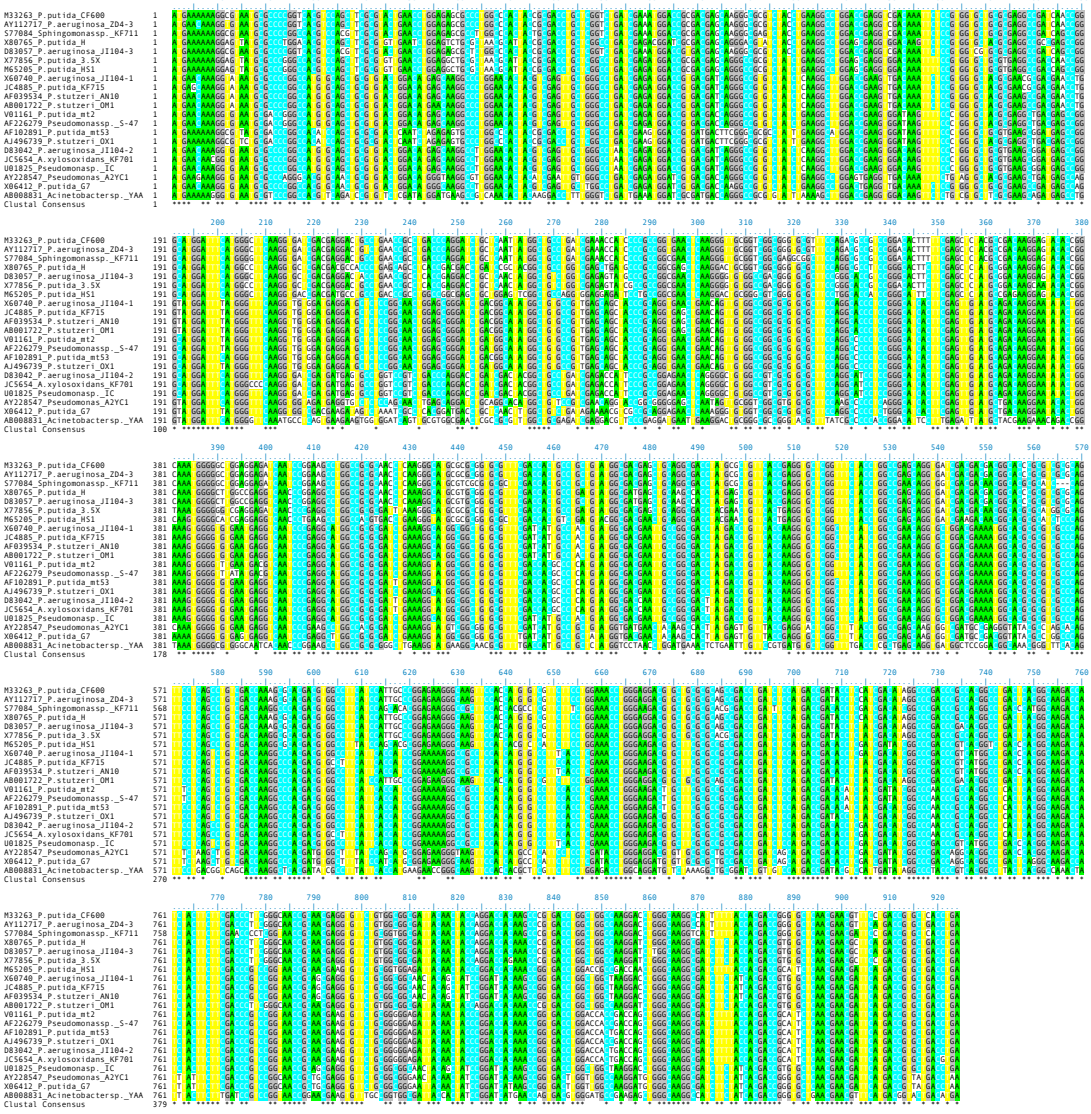
Supplementary online material

Supplementary online material 1.A Protein multiple alignment of Extradiol Dioxygenases Subfamily I.2.A genes used for primer design. Sequences names correspond to DDBJ/EMBL/GenBank accession number, organism of origin and strain designation.

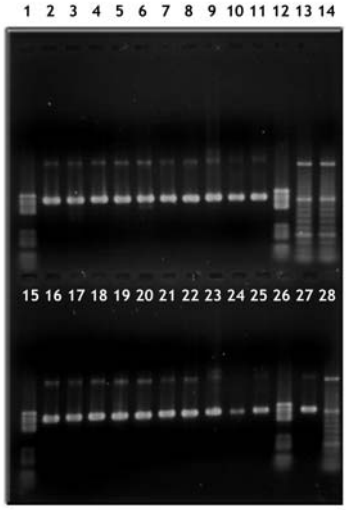


Supplementary online material 1.B

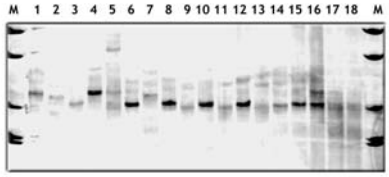
B. DNA multiple alignment of Extradiol Dioxygenases Subfamily I.2.A genes used for primer design. Sequences names correspond to DDBJ/EMBL/GenBank accession number, organism of origin and strain designation.



Supplementary online material 2PCR products obtained from reference strains, isolates and soil DNA using a primer set targeting a 527-bp fragment of C23O. Lanes 2 to 11, 16 to 25, and 27 contain respectively amplifications from *P. stutzeri* AN10, *P. putida* CF600, *P. putida* G7, *Pseudomonas* sp. IC, *P. putida* H (PG320), *P. putida* KT2440 plus pPGH11 (PG125 *P. putida* H), *E. coli* JM109 plus pCNU401 (*Sphingomonas sp.* KF711), *P. aeruginosa* JI104, *P. putida* mt-2, *P. putida* mt53, *P. stutzeri* OM1, *P. stutzeri* OX1, *P. putida* HS1, *P. putida* 35X from Shingler Lab, *P. putida* 3,5X from Hopper lab, Isolate A1YC1, Isolate A2YC1, Soil 1Y, Soil A3Y, Soil A1Y, and *P. putida* KT2440 TOL+. Any amplification was obtained in triplicates of negative controls (data not shown). Lanes 1, 12, 15 and 26 were loaded with 200 ng of molecular weight marker V (Roche), lanes 13, 14, and 28 were loaded with 250 ng of O´rangeRuler 50bp DNA ladder (MBI Fermentas).



Supplementary online material 3SSCP profiles of a 238b C23O fragment PCR amplified using as template DNA extracted from (lanes 1 to18) *P. putida* AN10, *P. putida* CF600, *P. putida* G7, *Pseudomonas* sp. IC, *P. aeruginosa* JI104, Sphingomonas sp. KF711, P. putida mt-2, *P. putida* mt53, *P. stutzeri* OM1, *P. stutzeri* OX1, *P. putida* H, *P. putida* HS1, *P. putida* 3,5X, Isolate A1YC1, Isolate A2YC1, equivalent mixture of all the previous fragments, Soil A1Y and Soil A2Y. Lanes M were loaded with 40 ng of molecular weight marker X (Roche)



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CHAPTER IV

Difference in kinetic behavior of catechol 2,3-dioxygenase variants predominant in a polluted environment[§]

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Difference in kinetic behavior of catechol 2,3-dioxygenase variants predominant in a polluted environment

4.1 Abstract

In a previous environmental survey of a polluted area (Junca, H., and D. H. Pieper. 2004. Environ. Microbiol. 6:95-110), two catechol 2,3-dioxygenase sequences predominant in environmental bacterial isolates and in contaminated soils were identified. We have cloned, sequenced and overexpressed the complete open reading frames by using information of stable operon arrangement, and conserved regions. A single amino acid substitution at position 218 had severe influence on enzyme kinetics, and the Tyr218 variant differed from the His218 variant by lower turnover number but higher affinity.

4.2 Text

Catechol and substituted derivatives are common intermediates formed from numerous natural and xenobiotic pollutants (Harwood and Parales, 1996; Reineke and Knackmuss, 1988; Smith, 1990) and can be subject to intradiol or extradiol cleavage, reactions which finally lead to mineralization. Predominantly extradiol ring-cleavage by catechol 2,3-dioxygenases is involved in the degradation of methylcatechols and examination of the evolutionary relationships (Eltis and Bolin, 1996) showed that the majority of catechol 2,3-dioxygenases were phylogenetically closely related, constituting the family of I.2 extradiol dioxygenases. Subfamily I.2.A seems to be of particular importance for the degradation of monocyclic aromatic compounds (Eltis and Bolin, 1996). Various mechanistic studies have been performed and the structure of an archetypal catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (Nakai *et al.*, 1983), has been elucidated (Kita *et al.*, 1999).

It is well known that the catalytic properties of catabolic enzymes can be dramatically influenced by single amino acid changes (Beil *et al.*, 1998; Parales *et al.*, 1999; Parales *et al.*, 2000; Pollmann *et al.*, 2003). Also in the case of members of the C23O subfamily I.2.A, significant differences in kinetic properties were observed in variants exhibiting few amino acid differences (Kitayama *et al.*, 1996; Williams *et al.*, 1990).

Usually, studies on regions affecting enzyme catalytic parameters were performed comparing isolates expressing different catechol 2,3-dioxygenases (Cerdan *et al.*,

1995; Kitayama *et al.*, 1996), by generation of chimeric proteins (Kikuchi *et al.*, 1999; Kitayama *et al.*, 1996) and/or selection of mutant proteins with new kinetic parameters under laboratory conditions (Cerdan *et al.*, 1995; Cerdan *et al.*, 1994; Kikuchi *et al.*, 1999; Wasserfallen *et al.*, 1991). One alternative approach to identify amino acids important to fine-tune catalytic activity is to recover natural diversity. In previous environmental studies we identified predominant C23O gene polymorphisms in BTEX contaminated environments (Junca and Pieper, 2003; Junca and Pieper, 2004). Samples heavily contaminated with benzene and toluene, contained an abundant C23O gene fragment (527 bp in length), identical in sequence with the C23O gene of *P. stutzeri* AN10, which differed from the otherwise predominant gene fragment by a single base (encoding a Tyrosine instead of Histidine at position 218), indicating that specific C23O genes and operons have been positively selected during the adaptation of soils to BTEX contamination. We had also observed, that out of 19 isolates harboring such C23O enzymes, only 2 isolates harboring the His218 variant could grow on benzene, toluene and ethylbenzene, whereas 17 isolates containing the Tyr218 variant could grow on benzene only. Moreover, whereas the His218 variant showed, among the substrates tested, the highest activity with catechol, the Tyr218 variant exhibited highest activity with 4-methylcatechol and a relatively elevated activity with 3-methylcatechol (Junca and Pieper, 2004).

To proof that specificity differences were actually due to variation in position 218, and not to the introduction of artificial mutations, the catechol 2,3-dioxygenases of two representative strains, *Pseudomonas* sp. strain 1YB2 ($C23O_{Tyr218}$) and *Pseudomonas* sp. strain 1YdBTEX2 ($C23O_{His218}$) were cloned by a new strategy. As the neighboring genes upstream and downstream of C23O sequences are relatively well conserved (Harayama *et al.*, 1987), primer sets annealing in conserved regions of the supposed operon neighboring genes encoding ferredoxin (e.g. *xylT* in *P. putida* mt2, *dmpQ* in *Pseudomonas* sp. CF600, (Hugo *et al.*, 2000) and 2-hydroxymuconic semialdehyde dehydrogenase (e.g. *xylG* in *P. putida* mt2, *dmpC* in *Pseudomonas* sp. strain CF600 (Harayama *et al.*, 1987; Shingler *et al.*, 1992)) were designed. Colonies were dissolved in 50 µl of water, boiled for 10 min (Kanakaraj *et al.*, 1998), centrifuged, and 4 µl of the supernatant were used as template for PCR reactions (50 µl) containing a final concentration of 1x PCR Buffer (Promega), 1.5 mM $MgCl_2$, 200 µM of each deoxyribonucleotidetriphosphate, 0.25 µM of each primer (forward primer FER2, 5' GCC YTG GCC TGY CRA STG TWT 3', and reverse primer MUCDOCB (5' TTC CAG GTC ATS AGC AGY AGC GG 3') (synthesized by Invitrogen) and 0.3 U/µl *Taq* DNA Polymerase (Promega). The PCR program was

as follows: one step at 94°C for 5 min, 10 cycles of touchdown PCR of 94°C for 1 min, 63°C (-1.5°C per cycle) for 1 min, and 72°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2.5 min followed by final elongation at 72°C for 8 min. To determine the correct size of the amplification fragments, the PCR products were run in 1.0% agarose gels (10 cm length, 1X TAE running buffer, 1.0 hour at 95 volts) and bands visualized by ethidium bromide staining (Sambrook *et al.*, 1989). Single bands of a size of approximately 1.7 Kb were observed, indicating the C23O genes to be located in operons similar to those of strains mt-2 and CF600. The PCR products were cleaned with Qiaquick PCR Cleaning Kit (QIAGEN), cloned in pGEM-T system and the ligation products transformed in *Escherichia coli* JM109 competent cells cultured according to manufacturer's instructions (Promega). Colonies were screened for expression of C23O by spraying with catechol (Junca and Pieper, 2004). Clones expressing C23O were purified and $C23O_{His218}$ or $C23O_{Tyr218}$ inserts from plasmids termed pC23Ohis218 and pC23Otyr218 were completely sequenced on both strands using the BigDye v1.1 system in an ABI 373A automatic DNA sequencer (Perkin-Elmer Applied Biosystems) with M13 forward and reverse primers (Sambrook *et al.*, 1989), and C23O inner primers described elsewhere (Junca and Pieper, 2004). The identity of $C23O_{His218}$ along the complete inferred protein sequence to the previously reported C23O gene carried by *P. putida* AN10 was confirmed, as well as the presence of only a single amino acid difference in $C23O_{Tyr218}$. By a similar strategy using primers annealing in neighboring genes, complete and functional genes could be isolated also directly from environmental DNA (data not shown). This straightforward strategy avoids the inherent introduction of artificial mutations by amplification of PCR products of primers annealing with mismatches inside the gene.

For overexpression of C23O enzymes, *Escherichia coli* JM109 (pC23Ohis218) expressing $C23O_{His218}$ and *E. coli* JM109 (pC23Otyr218) expressing $C23O_{Tyr218}$, were grown and cell extracts prepared as described previously (McKay *et al.*, 2003). For quantification of C23O variants in cell extracts, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad Miniprotein II essentially as described (Laemmli, 1970) with acrylamide concentrations for the concentrating and separating gels of 5 and 10% (wt/vol), respectively. Gels were stained using the fluorescent dye Sypro® Ruby (Molecular Probes Inc.) and scanned using a Fujifilm LAS-1000 CCD camera. A prominent band of 35 ± 2 Kda, which was absent in cell extracts of *E. coli* JM109 (pGEM-T) was observed in cell extracts of *E. coli* JM109 (pC23Ohis218) and (pC23Otyr218). The identity of the bands with $C23O_{His218}$ and $C23O_{Tyr218}$ was confirmed by N-terminal sequencing, and by MALDI-TOF

analysis as described previously (Junca and Pieper, 2004). The relative amounts of these C23O protein bands were determined using the AIDA 2.1 software package (Raytest Isotopenmessgeräte GmbH) as 29 ± 2 % of the total protein content in each case, showing that expression levels of the variants did not differ. Catalytic activities of C23O proteins were recorded on an UV 2100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Activities were determined in 50 mM K/Na-phosphate (pH 7.5) with catechol, 3-methylcatechol, 4-methylcatechol or 4-chlorocatechol as substrates, using extinction coefficients of reaction products previously described (Heiss *et al.*, 1995; Hirose *et al.*, 1994). V_{max}, k_{cat} and K_m values were determined using 1 - 100 µM of substrate and kinetic data were calculated from the initial velocities using the Michaelis-Menten equation by non-linear regression (KaleidaGraph, Synergy Software). As very low K_m values were indicated by this method, kinetic data were finally determined from progress curves obtained from reactions with initial substrate concentrations of 10 µM. Substrate concentrations during the reaction were calculated by the amount of ring-cleavage product formed during the further course of the reaction. Enzyme concentrations used (approx. 20 nM) ensure that less than 20 % of enzyme was inactivated during the reaction. As both extracts used contained about 29 % of C23O protein, it can be assumed, that the maximal transformation rate of $C23O_{His218}$ with catechol is approximately 4-fold that of $C23O_{Tyr218}$ (Table 1). Based on a subunit molecular weight of 35.050 Daltons, this corresponds to k_{cat} values of 128 s^{-1} and 38 s^{-1}, respectively. Less pronounced differences between the enzyme variants were observed for the turnover of substituted catechols, even though $C23O_{His218}$ usually exhibited higher turnover numbers. Thus, a dominant effect of the mutation is the increased turnover rate for catechol. In comparison to $C23O_{His218}$ and $C23O_{Tyr218}$, $C23O_{mt2}$ (Catechol 2,3-dioxygenase XylE, of *P. putida* mt2) showed k_{cat} values of 420 s^{-1} (Wasserfallen *et al.*, 1991) to 930 s^{-1} (Cerdan *et al.*, 1994) for catechol. A second difference between the variants is the significantly higher affinity of $C23O_{Tyr218}$ as expressed by the lower K_m values for all tested substrates.

C23O enzymes are characterized by their sometimes rapid inactivation through oxidation of active site iron during catalytic turnover. As an example the partition ratio (the number of substrate molecules consumed per number of enzyme

Table 1. Kinetic constants of catechol 2,3-dioxygenase C23OTyr218 and C23OHis218 a

Enzyme	Substrate	Activity (U/g)	k_{cat} (s^{-1})	K_m (µM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	Partition ratio[b]	J[c] ($10^{-3}s^{-1}$)	K_{mO2} (µM)
$C23O_{Tyr218}$	Catechol	64,700 ± 4,100	38 ± 2	1.1 ± 0.2	35	ND	ND	8 ± 2
	3-Methylcatechol	29,200 ± 2,000	17 ± 1	1.5 ± 0.2	11	4,000 ± 500	4.2	16 ± 4
	4-Methylcatechol	90,900 ± 7,300	53 ± 4	1.9 ± 0.2	28	6,200 ± 600	8.5	11 ± 3
	4-Chlorocatechol	29,400 ± 2,100	17 ± 1	0.9 ± 0.2	19	3,300 ± 400	5.2	17 ± 4
$C23O_{His218}$	Catechol	219,500± 14,100	128 ± 8	2.5 ± 0.2	51	ND	ND	10 ± 2
	3-Methylcatechol	42,800 ± 2,900	25 ± 2	3.2 ± 0.3	8	4,300 ± 500	5.8	40 ± 12
	4-Methylcatechol	109,900 ± 7,600	64 ± 4	5.2 ± 0.4	12	6,000 ± 600	10.7	14 ± 3
	4-Chlorocatechol	77,200 ± 5,800	45 ± 3	1.6 ± 0.2	28	6,800 ± 800	6.6	42 ± 10

[a] Experiments were performed with potassium phosphate buffer, pH 7.5 at 25°C. Kinetic parameters for catechols were measured in air-saturated buffer. J represents the apparent rate constant of inactivation of C23O by catechols. Kinetic parameters for oxygen were measured with saturating but noninhibiting concentrations of catechols. K_{mO_2} represent the Km for oxygen in the presence of the respective catechol cosubstrate.
[b] The partition ratio is defined as number of substrate molecules consumed/number of enzyme molecules inactivated.
[c] Values were calculated by dividing k_{cat} by the partition ratio (Vaillancourt *et al.*, 2002).

molecules inactivated) of $C23O_{mt2}$ with 4-ethylcatechol was reported to be 6,500, whereas that of 3-methylcatechol was 210,000 (Cerdan *et al.*, 1994). In a study relating kinetic properties of $C23O_{mt2}$ and variants with growth characteristics, Cerdan *et al.* (Cerdan *et al.*, 1994) assumed that a partition ratio of 18,000 may be a threshold allowing growth and a slight increase or decrease from the threshold may suffice to change the growth phenotype of hosts. Thus, partition ratios of $C23O_{His218}$ and $C23O_{Tyr218}$ were determined under saturating substrate concentrations (100 µM). The amount of enzyme (approx. 5 nM) was such that the enzyme was completely inactivated before 20% of the substrate was consumed. Whereas there was no significant difference in partition ratio between the variants (Table 1), the partition ratios observed with all three substituted catechols analyzed were dramatically lower than those observed for $C23O_{mt2}$ and even significantly lower than the assumed threshold value (Cerdan *et al.*, 1994). However, $C23O_{Tyr218}$ and $C23O_{His218}$, like $C23O_{mt2,}$ are linked with a ferredoxin encoding gene (XylT in *P. putida* mt-2), the function of which is the in vivo reactivation of C23O enzymes inactivated through oxidation (Hugo *et al.*, 1998; Polissi and Harayama, 1993) such that partition ratios lower than previously assumed, might allow growth.

The affinity for oxygen is another parameter critical for activity of catechol 2,3-dioxygenases and was determined from progress curves obtained from reactions with initial concentrations of catecholic substrates of 300 µM and 50 - 60 µM of oxygen. Substrate concentrations during the reaction were calculated by the amount of ring-cleavage product formed during the further course of the reaction.

Enzyme concentrations used (50 - 80 nM) ensure that less that 20 % of enzyme was inactivated during the course of the reaction. Both enzymes exhibited K_m values of 8 - 10 µM in the presence of saturating concentrations of catechol (Table 1). Those values are slightly higher than reported for $C23O_{mt2}$ (3.9 µM) (Kukor and Olsen, 1996). K_m values for oxygen in the presence of saturating concentrations of 3-methyl-, 4-methyl- or 4-chlorocatechol varied only slightly (by the factor of 2) in case of $C23O_{Tyr218}$. However, in comparison, K_m values for oxygen in the presence of saturating concentrations of 4-chlorocatechol and 3-methylcatechol were significantly increased in case of $C23O_{Tyr218}$. The influence of the substrate on the reactivity with oxygen was previously described for another type of extradiol dioxygenase, the 2,3-dihydroxybiphenyl 1,2-dioxygenases of *Burkholderia* sp. strain LB400 (Dai *et al.*, 2002) and *Rhodococcus globerulus* P6 (Vaillancourt *et al.*, 2003) and K_m values varied dependent on the chlorosubstituted dihydroxybiphenyl cosubstrate by one order of magnitude. According to the current knowledge on the mechanism of catechol cleavage by extradiol dioxygenases, the initial step is binding of the catecholic substrate to the ferrous center as monoanion (Bugg and Lin, 2001; Vaillancourt *et al.*, 2002) followed by binding of oxygen to the ferrous iron. Thus, the catecholic substrate will influence the binding kinetics of oxygen (Dai *et al.*, 2002).

Evidently, the nature of the amino acid at position 218 is severely affecting the enzyme kinetics. In previously described catechol 2,3-dioxygenase, this position is usually occupied by a histidine or phenylalanine and exceptionally a leucine (Fig. 1). A tyrosine in that position was only observed in a thermostable chimeric gene containing a central C23O gene fragment from an environmental sample (Okuta *et al.*, 1998). This position was, however, never considered as relevant for enzyme functioning. To understand possible influences of histidine/tyrosine variants on enzyme functions, three-dimensional models of these two protein types were constructed based on the available crystal structure of the highly homologous XylE protein ($C23O_{mt2}$) (Kita *et al.*, 1999). Amino acid 218 is neither involved in forming the substrate binding pocket nor interfering with the hydrophobic channel through which catechols and dioxygen are thought to enter the active site (Kita *et al.*, 1999). This amino acid is localized on the side of the molecule, which is buried in the tetramer interface and appears to be part of a second smaller channel leading to the active center. A functional role of this channel (Fig. 2) is unclear and His/Tyr 218, with a distance of more than 9 Å to the active iron and pointing away from it, is unlikely to interfere much with water or oxygen molecules filling the channel.

Figure 1. Alignment of 36 1.2.A C23O spanning 39 amino acids comprising residue 218 vicinity.

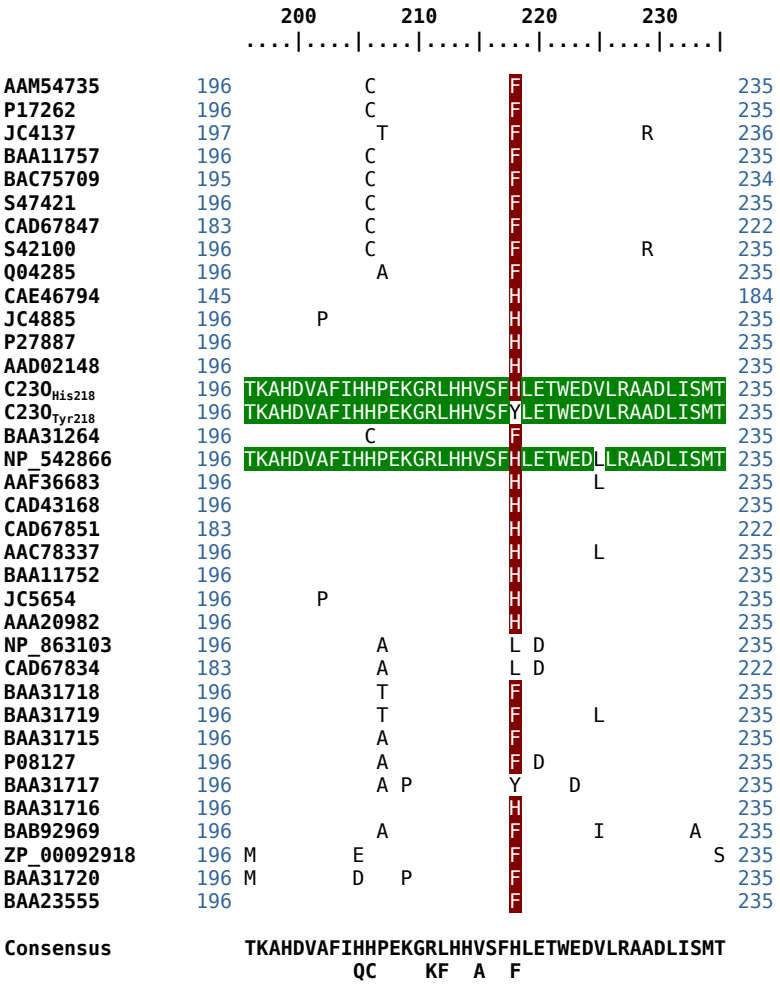

Fig 1. Alignment of 36 C23O subfamily I.2.A proteins. A selected block spanning 39 amino acids comprising residue the 218 vicinity is shown. Complete sequences used for the alignment (>60% identity) were obtained from GenBank/EMBL/DDBJ databases (respective accession numbers are given). Sequences are arranged (top to bottom) according to global alignment output (total similarity) using default values of the Clustal X (1.8) program. Amino acid sequences of *P. putida* mt-2 XylE (NP_542866), *Pseudomonas* sp. strain 1YB2 C23O ($C23O_{Tyr218}$) and *Pseudomonas* sp. strain 1YdBTEX2 C23O ($C23O_{His218}$) are shown (Grey-shaded white letters). Amino acids observed at the respective position in less then 20% of the sequences are indicated in the figure (black letters), whereas conserved amino acids or amino acids present at the respective position in at least 20% of the sequences are indicated at the bottom of the figure (consensus). Amino acids present at position 218 are shown for all the sequences. In this case, amino acids present in at least 35 % of the sequences are shown in black background and white letters.

Figure 2. Location of the His/Tyr218 relative to C23O active center.

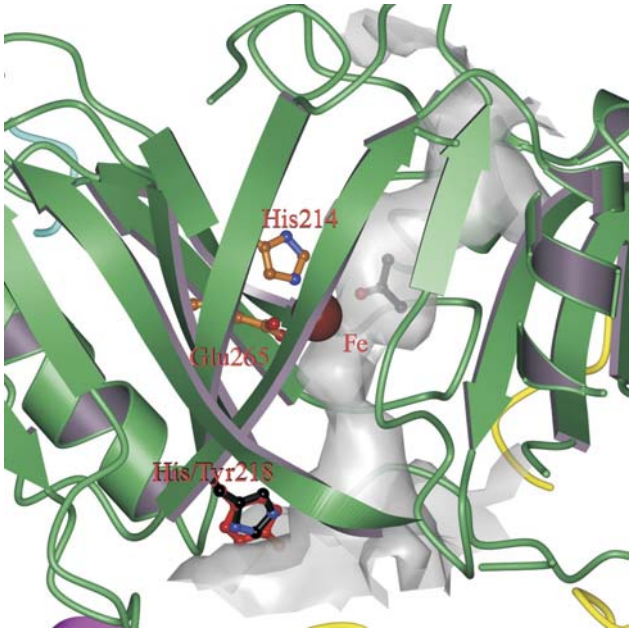

Fig 2. Location of the His/Tyr218 (black/red carbon atoms) relative to the active center of catechol 2,3-dioxygenase. In the active center the iron atom (brown sphere) with the bound His 214 and Glu 265 is shown. The substrate binding pocket is indicated by the acetone molecule observed in the catechol 2,3-dioxygenase structure 1MPY (Kita *et al.*, 1999) inside a semitransparent surface connected to the outside of the molecule on the top right. The His/Tyr 218 points to the tetramer interface (magenta and yellow tubes, bottom and right) at the entrance of a small channel leading from the interface to the active center. (Prepared with Molscript (Kraulis, 1991), GRASP (Nicholls *et al.*, 1991) and rendered with POV-Ray (TM), www.povray.org.)

Unfortunately, only a few previous studies have analyzed the effect of single amino acid mutations on the functioning of catechol 2,3-dioxygenases. Wasserfallen *et al.* (Wasserfallen *et al.*, 1991) screened for mutant $C23O_{mt2}$ allowing the *Pseudomonas* host to grow on 3-methylbenzoate in the presence of 3-chlorocatechol, a substrate mixture which the original host cannot deal with due to rapid inactivation of $C23O_{mt2}$ during 3-chlorocatechol turnover. A point mutation in *xylE,* which produced the substitution of Val291 for Ile291, enabled the host to grow on such mixtures. The mutant enzyme was characterized by decreased affinity for 3-chlorocatechol, increased affinity for 3-methylcatechol and higher partition ratios for substituted catechols, specifically 4-ethylcatechol. A set of $C23O_{mt2}$ mutants was isolated by a similar strategy (selection for growth on 4-ethylbenzoate) for their capability to transform 4-ethylcatechol (Cerdan *et al.*, 1994; Ramos *et al.*, 1987) and, like the Val291Ile mutant, Leu226Ser and Thr253Ile substitutions increased the catalytic reaction with 4-ethylcatechol. As the mutants exhibited reduced binding of the ferrous ion cofactor, it was assumed that the substitutions significantly modified the substrate binding pocket (Cerdan *et al.*, 1994). A further mutant Ala177Val was isolated based on its sensitivity towards 3-methylcatechol transformation, and a further Thr196Ile mutant reversed this effect. Astonishingly, none of the above mutations, all isolated based on a clear change of the phenotype of the host strain, was localized in the substrate binding pocket. Whereas the exchange of Thr253 to Ile can be assumed to result in a minor effect on the active site by abolishing a hydrogen bond participating in the stabilization of the Glu265 side chain, which is bound to the active iron, and Val291 is situated in the inner channel wall of the active site pocket (Kita *et al.*, 1999), Leu 226 and Ala 177 are situated near to the subunit interface, such that no direct influence of these amino aid on the active side can be envisaged. Similarly, a direct influence of a His to Tyr exchange at position 218 on the substrate binding pocket is not evident. However, various examples of mutant enzymes altered in their catalytic properties, hardly predictable by rational design, have been reported in studies on directed evolution of proteins. Only a careful analysis of structural changes of a set of mutants of a p-nitrobenzyl esterase identified networks of mutations that collectively remodel the active site (Spiller *et al.*, 1999) and it is a matter of fact that various proteins are altered in their kinetic properties by mutations in non-active site residues (Kraut *et al.*, 2003; Que and Ho, 1996; Shimotohno *et al.*, 2001; Zielinski *et al.*, 2003). Some mutations may not alter the average structure of the protein as determined by crystallography but may exert an effect on enzymatic activity of the protein by changing its dynamics (Yang *et al.*, 2003) or create small structural changes

effecting protein kinetics by long-range interactions, which are difficult to rationalize.

Interestingly, there are several characteristics distinguishing the natural occurring Tyr218 and the His218 variant. The Tyr218 variant, detected both in highly and slightly contaminated soil, and present in benzene degraders exhibits low turnover number and high affinity, whereas the His variant, detected in highly contaminated sites only and present in toluene/benzene degraders, exhibits high turnover numbers and low affinity. As such, the variants seem to be selected for by the environmental conditions. If the different growth phenotype of strains harboring the Tyr218 and those harboring the His218 variant is actually due to the different kinetic characteristics of the enzymes will be evaluated by site directed mutants.

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CHAPTER V

General Discussion

Improved tools to determine shifts in functional gene diversity: from fitness in communities to sequence polymorphisms affecting activities

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General Discussion

Improved tools to determine shifts in functional gene diversity: from fitness in communities to sequence polymorphisms affecting activities

Developments in molecular techniques have led to rapid and reliable tools to monitor microbial community structures and dynamics under *in-situ* conditions. However, even though various functional genes are localized on mobile genetic elements such that metabolic potential/activity is not necessarily reflected by the community structure, there has been a lack of emphasis on monitoring functional diversity. A more detailed picture of the catabolic gene structure and sequence diversity in environmental samples will significantly increase our knowledge of the functional potential of microbial communities. We adapted suitable techniques to follow functional gene diversity and applied those to catechol 2,3-dioxygenases as key genes in aromatic hydrocarbon degradation.

5.1 Generation of strain collections as culture dependent background

A new method for isolating strains capable of growing on BTEX compounds was developed to diminish pre-selection or enrichment bias and to assess the function of predominant gene polymorphs. Selection was based on the isolation of microorganisms capable to express C23O as key enzyme of aerobic aromatic degradation. This isolation resulted in a diverse collection of organisms able to use as a sole carbon and energy source at least one of the BTEX compounds, and all the degraders could use benzene as a sole carbon and energy source. To our knowledge, this is the first collection of benzene degraders from the environment. There was a strong correlation between BTEX degradation and presence of a C23O, specifically of a subfamily I.2A C23O. The strain collection contained a broad set of organisms with unusual degradation profiles, among them strains capable to degrade benzene but not toluene, despite the presence and induction of a C23O. From a simple biochemical consideration, such growth profile could have two reasons, the absence of a C23O and degradation of benzene via the 3-oxoadipate pathway (Reineke, 1998), which is not suited for degradation of methylaromatics (Rojo *et al.*, 1987), or the absence of a 2-hydroxymuconic semialdehyde hydrolase, indispensable for growth on toluene via 3-methylcatechol (Harayama *et al.*, 1987; Sala-Trepat *et al.*, 1972). Such an absence was not expected based on the known structures of *meta*-cleavage pathway gene operons, and was not the reason for the

new phenotype, as all strains analysed in this aspect express a functional 2-hydroxymuconic semialdehyde hydrolase.

The isolates showing the unexpected benzene but not toluene mineralising phenotype harboured C23O genes, identical to the gene variant predominant in all contaminated sites analysed. Thus, considering the abundance of this gene variant in the culture collection and the strong correlation with the unexpected phenotype, it can be suggested that this variation has a strong influence on metabolism and phenotype. Such influences of slight changes in kinetic properties of catechol 2,3-dioxygenase gene variants on the catabolic phenotype have previously been observed for the degradation of 4-ethylbenzoate (Ramos *et al.*, 1987), 3-methylbenzoate (Cerdan *et al.*, 1994) and mixtures of 3-methylbenzoate and 3-chlorocatechol (Wasserfallen *et al.*, 1991). Isolates harbouring a C23O gene variant differing by a single point mutation and observed in highly polluted sites only, were, in contrast, capable (among some other isolates) to mineralise benzene and toluene, indicating a catabolically determined sharing of carbon sources between groups harbouring different catabolic gene variants on-site.

5.2 General considerations for catabolic profiling of environmental communities

The culture independent analyses of C23O genes in previously reported studies was usually based on the use of primers designed for discrete detection (presence/absence of the expected amplification fragment) (Mesarch *et al.*, 2000; Meyer *et al.*, 1999; Okuta *et al.*, 1998; Wikstrom *et al.*, 1996). Whereas some primers could successfully be used for amplifying C23O gene fragments from reference strains (Mesarch *et al.*, 2000), they are definitively not useful for amplifying from soil DNA extracts, possibly due to mispriming and amplification of DNA other than the target. Careful selection of primers proved to be absolutely necessary for any environmental application.

The development and validation of a MPN-PCR assay to estimate quantities of the target gene , is a rapid and inexpensive strategy when many environmental samples need to be analysed. Only few reports deal with the direct quantification of functional genes in environmental samples (Baldwin *et al.*, 2003; Ringelberg *et al.*, 2001). Such a quantification is extremely useful to understand the fitness or relative importance of a character, helping to select catabolic genetic indicators to determine specific responses in the community. Even more, MPN-PCR was successfully coupled with restriction fragment analysis (AFDRA to analyze gene phylogeny), allowing the rapid detection of predominant polymorphisms. Thus,

not only quantitative information on the fitness of a gene family in the environment is retrieved, but also on the specific gene variant conferring such advantage. This insight into the direct link between adaptation and function, (see Chapter IV), helps to direct the efforts to analyze selected catabolic gene variants in the environment.

With the primers designed in this work, it was possible to generate profiles of C23O subfragments of sizes close to the upper size limit (600 bp) of the SSCP method. The advantage of using sizes close to the maximum length resolved by fingerprinting methods like SSCP, TGGE or DGGE, is that such subfragments provide sequence information (around 500 bp) on more than 50 % of the C23O gene targeted here, allowing the generation of reliable gene phylogenies. It thus seems generally to be possible to obtain sequence information of significant parts of various families of functional genes by fingerprinting after careful selection of primers to be used for amplification.

As evidenced for C23O genes, such primers can be used to determine, after fingerprint generation the most abundant gene sequences in the PCR amplicons, The results of the fingerprinting methods were validated by random sequencing of clone libraries. Such a careful assessment, if the information obtained from fingerprints is representative for the environment analyzed, seems to be of general importance. In contrast, several reports used PCR fragments to produce gene fingerprints from complex mixtures (Duarte *et al.*, 2001; Felske *et al.*, 2003; Oved *et al.*, 2001; Watanabe *et al.*, 1998) apparently without previous analyses about how accurately those fingerprints are reflecting the diversity of the amplicon, or, in case of restriction patterns, how accurate the pattern is reflecting the sequence differences or phylogeny (Bakermans and Madsen, 2002; Tan *et al.*, 2003). Moreover, a careful assessment on how informative the diversity of a given fragment is in terms of functional properties, is necessary, if intensive application of fingerprints for similarity comparison between many samples is intended. However, with the current knowledge, only in some cases a sequence information can be directly related with a detailed function and it remains to be elucidated if gene fragments derive from functional genes and if sequence diversity is related to functional diversity.

Regarding functional analyses of C23O PCR fragments from the environment, Okuta *et al.* (Okuta *et al.*, 1998) used fragments from soil to assemble them into a well described C23O gene and to screen for new catechol 2,3-dioxygenase activities, in that case thermostable variants. Interestingly, primer sets designed thus far for C23O genes, comprised only fragments of genes. We report for the

first time the use of conserved gene arrangements to amplify unknown complete C23O functional genes. This strategy was used to overexpress C23O from isolates, but can similarly well be used to amplify complete and functional C23O genes from environmental DNA. Given the fact that even the availability of crystal structures does not allow to predict structure/function relationships, and that small changes in the protein, even outside of the substrate binding pocket, can significantly influence kinetic properties (Kraut *et al.*, 2003; Que and Ho, 1996; Shimotohno *et al.*, 2001; Zielinski *et al.*, 2003), the recovery of complete functional genes from the environment permits to obtain kinetic information on predominant gene variants, to recover genes with new kinetic properties, and to upgrade information on structure/function relationships in environmentally important catabolic activities.

This strategy seems to be not only applicable to C23O genes, but to a diverse collection of other genes coding for key activities. As an example, phenol hydroxylases and benzene/toluene monooxygenases encoding gene clusters are highly conserved in gene order (Colby and Dalton, 1978; Miura and Dalton, 1995; Newman and Wackett, 1995; Pikus *et al.*, 1996; Powlowski and Shingler, 1994; Small and Ensign, 1997). Like in Rieske type non-heme iron dioxygenases, only certain subunits of the enzymes are responsible for substrate specificity, as they comprise the active site. In muticomponent phenol hydroxylases (Canada *et al.*, 2002) and benzene/toluene monooxygenase, like in multicomponent dioxygenases (Beil *et al.*, 1998; Erickson and Mondello, 1993; Gibson and Parales, 2000; Parales *et al.*, 1998; Zielinski *et al.*, 2003), the α-subunits affect substrate specificity (Mitchell *et al.*, 2002; Pikus *et al.*, 1997), whereas other subunits of the oxygenase complex either act as connectors to maintain subunit structure or are responsible for electron transport, and therefore of minor importance for substrate specificity (Leahy *et al.*, 2003). However, in benzene/toluene monooxygenases, also the effector protein seems to be important for selectivity (Mitchell *et al.*, 2002). Benzene/toluene monooxygenases will significantly shape metabolic nets in communities based on their regioselectivity and thus pathway selection. Complete α-subunits can be assumed to be extracted from the environment by designing primers based on neighbouring genes. As it had been previously reported for dioxygenase systems (Armengaud and Timmis, 1997), selection systems can be designed based on incomplete gene clusters comprising other subunits, which are only active when they are complemented with the subunit determining substrate specificity.

The above described methods rely on nucleic acid probes and PCR primers designed based on information retrieved from isolates and can thus only cover a

subset of the activities assumed to be present in environmental samples and will not cover new genes or gene products. In order to access the full genetic diversity able to catalyse a certain type of reaction, it is necessary to employ function-based screens. One approach that does not rely on conserved nucleotide sequences is to use genomic libraries to retrieve genes from natural bacterial communities without cultivation. Successful reports on the construction of environmental DNA libraries and isolation of genes encoding a certain property have recently been published (Henne *et al.*, 1999; Knietsch *et al.*, 2003; Lorenz *et al.*, 2002; Lorenz *et al.*, 2003; Schloss and Handelsman, 2003). For understanding the metabolic network in microbial communities, it is thus important to combine function based screens with culture independent surveys of genes encoding functional properties.

5.3 Linking community composition and function

Even though the community composition in the BTEX contaminated sites analysed has not been determined by culture independent methods, characterization of isolates from highly and low contaminated sites gave indications that the community structure between those sites differs. Whereas BTEX degrading isolates from the low contaminated site comprise mainly organisms closely related to *P. brassicacearum* NFM421, the culture collection from the highly contaminated site comprise mainly organisms closely related to *P. fluorescens* DSM 50108. However, there was no indication on metabolic properties to be related with the taxonomic position of the strains. It more seems that different organisms with nevertheless identical metabolic properties were present in the differently contaminated environments, and that *P. fluorescens* strains were favored by the general environmental conditions of the highly contaminated environment. It moreover seems that C23O genetic determinants are localized on mobile genetic elements and spread among different recipients.

It should be noted in this context, that genetic determinants of catechol *meta*-cleavage pathways were not detected in any of the thus far reported *Pseudomonas* spp. genome sequencing projects (Jimenez *et al.*, 2002; Nelson *et al.*, 2002; Stover *et al.*, 2000), and are predominantly found on plasmids (Bartilson *et al.*, 1990; Carrington *et al.*, 1994; Herrmann *et al.*, 1995; Schell, 1983; Voss *et al.*, 1990; Williams and Murray, 1974).

As an example, the TOL plasmid had been transferred by conjugation to different species (Calero *et al.*, 1989; Mäe and Heinaru, 1994; Ramos-Gonzalez *et al.*, 1991; Ramos-Gonzalez *et al.*, 1994; Smets *et al.*, 1994) and transfer of the TOL plasmid was also observed in natural ecosystems. In fact, the TOL plasmid has been used

as a marker to estimate DNA conjugation or retrotransfer in soils (Molbak *et al.*, 2003; Nancharaiah *et al.*, 2003; Ronchel *et al.*, 2000).

Moreover, catabolic genes are often localized on transposons (Tan, 1999; Top and Springael, 2003). As an example, the transposons Tn4651 (56 kb) and Tn4653 (70kb), with the latter including the former, carry all the *xyl* toluene degradation genes from plasmid pWW0 (Tsuda and Iino, 1987; Tsuda and Iino, 1988), and *xyl* genes of pWW53 are located on Tn4653 (39 kb) (Tsuda and Genka, 2001). Both Tn4651 and Tn4563 or similar mobile elements are assumed to be responsible for the reported transpositions of *xyl* gene clusters into the chromosome (Assinder and Williams, 1990; Sinclair and Holloway, 1991; Sinclair *et al.*, 1986) of bacteria or resistance or catabolic plasmids (Jahnke *et al.*, 1993). In addition, the chromosomally encoded *meta*-cleavage pathway genes found in *P. putida* P35X and *P. stutzeri* AN10 (Bosch *et al.*, 1999; Ng *et al.*, 1994) suggest a recruitment by transposition events.

Even though the transfer of catabolic genes by conjugation and transposition is for sure an important factor explaining the rapid evolution of bacteria to degrade pollutants (Muller *et al.*, 2003), also natural transformation among microorganisms is involved in bacterial evolution. Importantly, *P. fluorescens* has been reported to undergo transformation in soil microcosms without any specific physical or chemical treatment (Demaneche *et al.*, 2001).

The results in the current thesis of different populations carrying out similar tasks in different environments is not astonishing. Similarly, Wilson (Wilson *et al.*, 2003) described naphthalene degrading organisms from a hillside soil to be different from those of the more contaminated seep sediments. When the information of 16S rRNA gene sequences and catabolic dioxygenase *phnAc* sequences were determined from these isolates, the divergence at taxonomical level was greater than the divergence at the catabolic gene level. It was suggested that a highly conserved gene is shared by a group of taxonomically different hosts, and that horizontal transfer of the *phnAc* gene had taken place among the community members.

5.4 Catabolic gene variants selected in the environment as a way to elucidate sequence/function relationships

Two strains representing predominant catabolic phenotypes and harboring C23O gene variants differing by a single amino acid residue had been subjected to a detailed study and significant differences in the kinetic performance of the variants were visible. The observed variation was localized on the side of the molecule,

which is buried in the tetramer interface and appears to be part of a small channel of unclear functional role leading to the active center. Like for other variants of catechol 2,3-dioxygenases described previously (Cerdan *et al.*, 1995; Wasserfallen *et al.*, 1991), a direct influence of the amino acid on the substrate binding pocket is not evident and small structural changes effecting protein kinetics by long-range interactions seem to be the reason for differences in kinetic properties. Thus, our study is an example of natural "breeding" of enzyme variants with new unexpected properties, which have not been accessible by rational design.

Whereas the difference in enzyme kinetics of the analyzed variants was drastic in case of catechol transformation, slight effects were observed considering 3-methylcatechol transformation. Thus, a direct clear-cut link between catabolic activity of a key enzyme and catabolic phenotype is not visible. A final proof, that the difference in catabolic pohenotype is really due to different kinetic properties can only be performed by directed mutagenesis studies of a one variant, such that any influences of other catabolic genes are excluded.

Thus far, some more information on benzene degradation in both strains analyzed has been accumulated. C23O, 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase, and a benzene-*cis*-dihydrodiol dehydrogenase are constitutively expressed, suggesting a very similar operon composition and conservation in both strains. The metabolic pathway of benzene can be assumed to proceed by dioxygenation to form a benzene-*cis*-dihydrodiol, which is dehydrogenated to catechol. Gene fragments of toluene/benzene dioxygenases (524 bp), were amplified and sequenced from both catabolic phenotypes, and the sequences exhibited >95 % identities in the putative translated peptides. At first sight, the obvious amino acids differences are not expected to have influences on the catabolic phenotype. However, from the experimental work described above, such predictions have to be carefully analyzed.

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CHAPTER VI

Outlook

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OUTLOOK

In this study, C23O genes were successfully used to validate molecular methods to follow functional gene diversity, thanks to their subfamily sequence conservation, culturability of representative hosts and the ease of screening for this activity. However, to get a detailed insight into the metabolic net of microbial communities, additional catabolic targets should be assessed, together with analysis of taxonomical compositions, to deduce function/structure relationships at the community level. It had previously been indicated (Stapleton and Sayler, 1998) at the natural attenuation test site (NATS), Columbus Air Force Base, MS, that the indigenous microorganisms adapted to the contaminant hydrocarbons BTEX and naphthalene exhibited increases in gene dosage of the targeted genotypes *nahA*, *nahH*, *todC1C2*, and *xylA*, suggesting that an aerobic contaminant-degrading community successfully developed within the plume. Other studies indicated increase in gene dosage of specifically ring-activating monooxygenase genes under increased aromatic contamination (Baldwin *et al.*, 2003; Ringelberg *et al.*, 2001). It is thus not clear which factors influence to what extend the metabolic of aromatic degradation under *in situ* conditions, mainly because no systematic study on catabolic diversity and abundance has been performed thus far.

During the course of this experimental work, primers targeting conserved regions in the α-subunits of toluene dioxygenases, benzene/toluene monooxygenases as well as phenol hydroxylases had been designed and tested in type strains. The conditions to generate PCR-SSCP profiles have been optimised to generate reproducible catabolic gene fingerprints of each gene type from DNA extracted from soils samples of the site under study (Chapter I, Fig. 14).

To determine taxonomical and functional shifts in microbial communities under adaptation to BTEX an on-site experiment was conducted in collaboration with the AMICO project partners VITO and AQUATEST. Soil samples from the uncontaminated area were introduced into membrane bags made of inert material (the pore size in the mesh used to construct these bags contained the soil particles but allowed bacterial cell and nutrients exchange), and placed back into the site in two distinct places, at the non contaminated site of origin (A) and at the site highly contaminated with BTEX (B). To be capable of distinguishing between adaptation processes and colonization, a sterile soil sample was introduced into the highly contaminated area (C). Samples were collected consecutively during 4 months, and used for culture dependent and culture independent analyses.

In figure 1 the catabolic gene profiles obtained by PCR-SSCP of toluene dioxygenases, phenol hydroxylases, and catechol dioxygenases (C23O) are shown, as well as PCR-SSCP profiles of 16S rRNA as an indicator for taxonomical structure. The community structure in the soil sample reintroduced into the site of origin showed significant changes over time, whereas the catabolic gene composition was rather stable. Changes in catabolic gene profiles were only observed for toluene dioxygenases. Obviously environmental factors (possibly seasonal variations) other than aromatic pollution, are shaping the community at that site.

When microcosms were placed into the heavily contaminated site, a rapid response and change in microbial community structure was observed and the community composition remained stable throughout the course of the experiment. Highly similar profiles were observed in case sterile microcosms were used, indicating a rapid colonization of the microcosm by the (probably adapted) microbial community of the heavily contaminated site.

It is important to remark that profiles of all the catabolic genes could be generated, even in the absence of pollution, indicating such catabolic genes to be widespread. However, it is directly evident, that catabolic genes predominant in the clean environment are different from those at the contaminated site. Thus, there it not only, as previously observed (Margesin *et al.*, 2003; Ringelberg *et al.*, 2001; Siciliano *et al.*, 2001; Wikstrom *et al.*, 1996), a change in abundance of members of specific gene families, but also a drastic change in their composition. Therefore, analysis of the polymorphism selected under high BTEX concentrations in the community could provide hints on adaptations (selection of catabolic genes previously present in low amounts in the soil) that could be functionally important for the effective survival of the hosting species selected, and for effective biodegradation of BTEX. This nearly immediate adaptation observed by molecular fingerprints requires deep investigation of sequence information as it had been performed for C23O profiles. Furthermore, cross comparison between 16s rRNA fingerprints and catabolic gene fingerprints would help to understand the relationships between taxonomical and functional adaptations in natural microbial communities under pollutant stress.

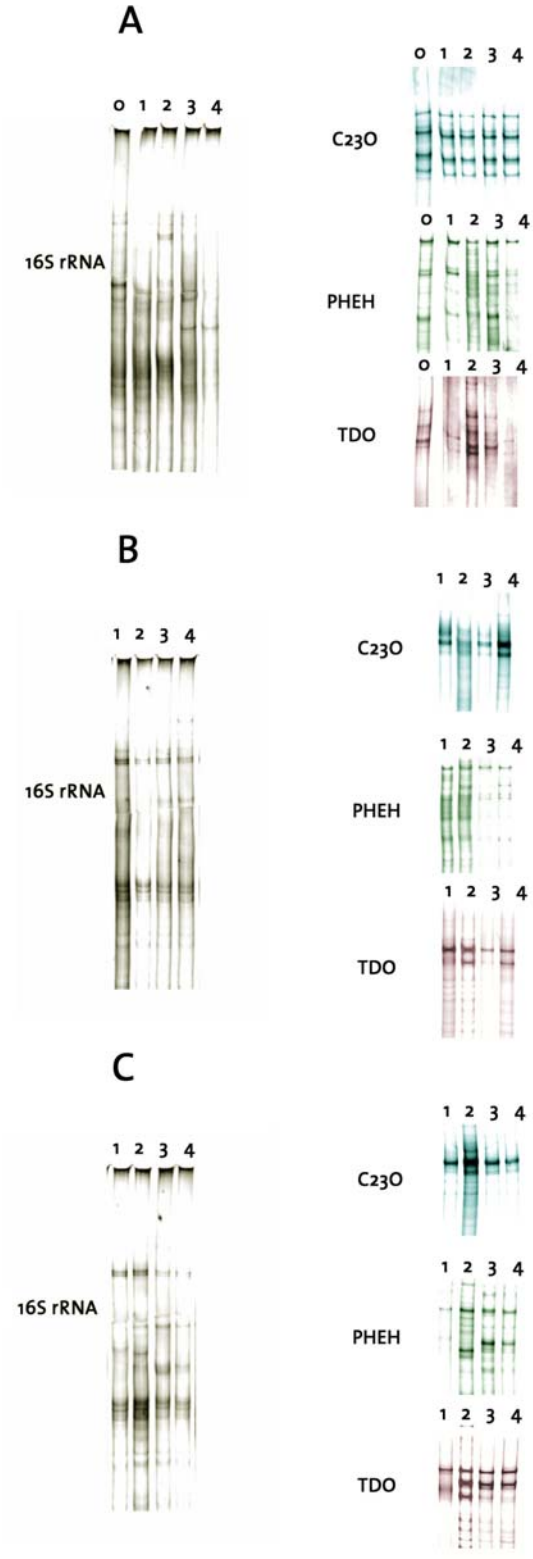

Fig. 1. PCR-SSCP using the single strand removal approach (Schwieger and Tebbe, 1998) of 16S rRNA, catechol 2,3 dioxygenases (C23O), three-component phenol hydroxylases/toluene monooxygenases (PHEH), and toluene dioxygenases (TDO) gene fragments, from soil samples under *in situ* adaptation to BTEX at different times (for description, see main text).
A) Clean soil reintroduced to the clean source site. B) Clean Soil introduced at the highly BTEX contaminated area
C) Sterile soil introduced at the highly BTEX contaminated area.
1 to 4 correspond to soils sampled after 1 week, 3 weeks, 6 weeks and 16 weeks of *in situ* incubation, respectively. The fingerprints of the soil sample used to fill the bags are indicated with 0

The quantification and fingerprinting of functional genes from environmental DNA will identify those selected in the community. However, such methods are thus far applied to DNA rather then mRNA. Optimization of mRNA extraction from environmental samples has still to be optimized in order to be capable to analyze

genes which are activated under a given environmental condition (Alfreider *et al.*, 2003; Corkery and Dobson, 1998; Meckenstock *et al.*, 1998; Weinbauer *et al.*, 2002; Wilson *et al.*, 1999). Moreover, a better link is needed between functional and taxonomical diversity. Such a link between community composition and community function can be made by stable isotope probing (Jeon *et al.*, 2003). Stable isotope probing using labelled substrates has been successfully used to characterize active members in biodegradation or denitrification processes (Ginige *et al.*, 2004; Hutchens *et al.*, 2004; Lueders *et al.*, 2004; Manefield *et al.*, 2002; Radajewski *et al.*, 2000; Radajewski *et al.*, 2003; Wellington *et al.*, 2003) by analyses of nucleic acids (16S rDNA or rRNA) becoming labelled during metabolism. A logic future trend would be the generation of both taxonomic and catabolic fingerprints from the labelled nucleic acids, which would give very precise information on the community members efficiently degrading and incorporating the substrate carbon into biomass and nucleic acids, as well as active pathways. However, as all PCR based methods such analysis will only give information on gene families and functional properties known from isolates, these methods should be supported by culture-independent activity-based screenings (function-driven analyses) of *meta*-genome libraries (Schloss and Handelsman, 2003). Consistently, in the future, also more efforts could be directed to this search of functions of genes overlooked by similarity, in parallel to studies filling the gaps in knowledge on structure/function predictions of the already known homologous gene families. As an example, it was believed for a long time that degradation of toluene should be initiated either by dioxygenation (Gibson, 1968; Gibson *et al.*, 1970; Gibson *et al.*, 1968) or oxidation of the side chain (Worsey and Williams, 1975), and only later research revealed that various microorganisms initiated degradation by monooxygenation (See Chapter I, Section 1.7.2). Angular dioxygenation of dibenzofuran and dibenzo-*p*-dioxin (Armengaud *et al.*, 1998) was initially thought to be a capability of certain members of the benzene/toluene/biphenyl group (Gibson and Parales, 2000; Nam *et al.*, 2001) of dioxygenases, however, recent studies indicated various angular dioxygenases to belong to the phthalate family of dioxygenases (Kasuga *et al.*, 2001; Sato *et al.*, 1997). Salicylate 1-hydroxylation was thus far thought to be catalyzed exclusively by single component flavoproteins, but recently multicomponent salicylate 1-hydroxylases were reported (Pinyakong *et al.*, 2003), and even the direct ring-cleavage of salicylate was described (Hintner *et al.*, 2001). The metabolic diversity of microorganisms is by far broader than currently known from studies of isolates and to access such broad diversity evidently needs function based screens to

complement molecular ecology surveys of known family members. Even in the case of extradiol dioxygenases, our knowledge on functional and phylogenetic diversity is still fragmentary. In the recent years, various new members of this family have been reported, expanding our knowledge on the diversity of this family, filling the protein space and evolutionary gaps expected (Voigt *et al.*, 2000), as it would be predicted for a specific protein family (Naylor and Gerstein, 2000). Various of these proteins would not have been detected based on previous consensus sequences, as they represent evolutionary divergences. In figure 2, a comparison between knowledge on extradiol dioxygenase family phylogeny in 1996, and in 2003 is shown. Many of the sequences used to reconstruct the evolutionary relationships of extradiol dioxygenases are not fully characterized at functional level. Therefore, gene sequences are still the criteria to define subfamilies, and only general relations with functions can be drawn, which are often oversimplifications. For example, the single gene representing family I.5 was shown to encode a Mn-dependent dioxygenase (Boldt *et al.*, 1995), suggesting that related proteins would have structural features necessary for Mn-dependent enzymes. However, a highly homologous enzyme (Wang and Lipscomb, 1997) has been now identified to be Fe-dependent. Based on the functional analysis of three subfamily members, subfamily I.2.C has been suggested to comprise enzymes characterized by increased affinity for oxygen (Kukor and Olsen, 1996), however, it is not clear if this is actually true for the recently identified closely related enzymes. In addition, various extradiol dioxygenase-like gene sequences only distantly related to previously reported catechol 2,3-dioxygenases were obtained by genome sequencing projects, and functional characterization is missing.

An increased representation of new members (sequence diversity) inside catabolic gene families, is observed in all the families mentioned in this work, however, functional analyses are missing in most cases.

However, even with detailed knowledge on a given protein and the availability of structural data, the effect of small sequence changes on catabolic performance cannot be easily predicted. Our results are showing that it is not sufficient to simply compare sequences retrieved, translate the information to a putative protein sequence, and create structural models to infer the possible effects of polymorphisms on function but to validate information by functional analyses.

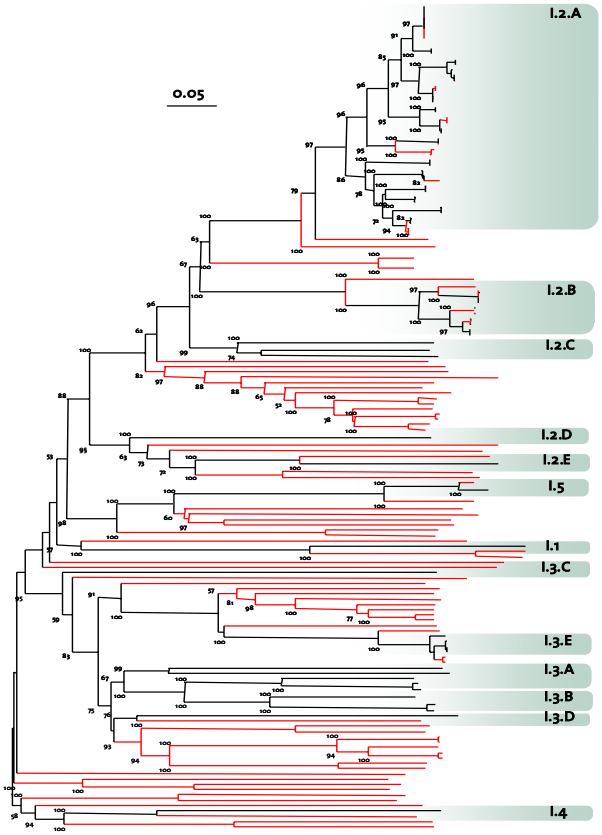

Fig. 2. Extradiol dioxygenases phylogeny 1996 –2003. Sequences used to delimit families and subfamilies for extradiol dioxygenases in 1996 (Eltis and Bolin, 1996) are represented as black lines. New family members detected and retrieved from EMBL/GenBank/DBBL databases(November 2003) are represented with red lines.

The functional analysis of gene variants selected in nature (from isolates or directly obtained from the environment by PCR clone libraries or fingerprint screening and sequencing) will without doubt increase our knowledge on structure/function relationships and adaptation of environmental metabolic nets to new challenges but also will result in the identification of new enzymes of biotechnological importance. Thereby, ecological research meets with research on directed evolution of enzymes, which has emerged as a popular method for protein engineering (Hall, 2002; Stemmer, 1994). Mimicking natural evolution, an initial parent gene (or genes) is chosen and a diverse library of variant genes is created through mutagenesis or recombination or through *de novo* generation by combination of peptides (Wei *et al.*, 2003) or oligonucleotides (Zha *et al.*, 2003). Directed evolution often discovers improvements by making a few amino acid substitutions that collectively have an important functional effect, without the necessity of structural data. In this way our knowledge of those amino acid positions altering kinetic parameters in catabolic enzymes can be significantly increased.

Certainly, the phenomenon of single amino acid changes having severe effects on biodegradation performance should be considered as a critical factor when trying

to perform modelling of biodegradation networks. The assumption that the merely presence or absence of a gene in a bacterial genome or community metagenome, could be simplified in computational models as determinant of metabolite flux, and then theoretically channelled into a discrete direction, would lead to severe experimental pitfalls. A number of recent reports are showing such *in silico* 'metabolic reconstruction' as a valid way to infer biodegradation potential of organisms with a certain genome or the evolution of catabolic pathways (Ellis *et al.*, 1999; Pazos *et al.*, 2003). However, it should be kept in mind that single amino acid changes not only effect enzyme kinetics. More severely, single amino acid differences are known to change the substrate binding pocket in a way that new substrates can be degraded (Beil *et al.*, 1998). Changes in regioselectivity of oxygenation of aromatics exerted by single amino acid differences can result in channeling of a substrate in completely different metabolic routes (e.g. degradation of toluene after *ortho*-monooxygenation proceeds via 3-methylcatechol, whereas para-monooxygenation can trigger channelling of the substrate into the protocatechuate pathway) or result in formation of dead-end products.

It is thus clear that the current knowledge on enzyme kinetics and interaction of multiple aromatic substrates has to be upgraded and integrated into modelling approaches to achieve accurate and reliable predict of biodegradation network based on genome information (Hua *et al.*, 2003).

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Apendix

Nucleic acid sequences determined during the course of this doctoral thesis, columns up to down, left to right.

(Note: in the PDF version of this document, each accession number has the corresponding hyperlink to the GenBank/EMBL/DDBJ databases)

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  TITLE     Functional gene diversity analysis in BTEX contaminated soils by
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  JOURNAL   Unpublished
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      121 gcatggcggc tgtgcgtttc gatcattgcc tgctgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545010               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_G_51_A3Y.
ACCESSION   AJ545010
VERSION     AJ545010.1  GI:28556824
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545009               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_C_8_A3Y.
ACCESSION   AJ545009
VERSION     AJ545009.1  GI:28556821
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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      121 gcatggcggc tgtgcgtttc gaccattgcc tgctgtatgg cgacgaactg caggcgacct
      181 atgacctgtt caccaaggtg ctcggtttct atctggccga gcaggtggtg gacgacaacg
      241 gcacgcgcat cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545008               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_A_14_A3Y.
ACCESSION   AJ545008
VERSION     AJ545008.1  GI:28556819
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ORIGIN
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      121 gcatgcgcgc ggtgcgtttc gaccactgtc tgctgtacgg cgacgagctg caggcgacct
      181 acaatctgtt caccgaggtg ctcggtttct atctggccga gcaggtggtc gacgacaacg
      241 gtacgcgcat tgcgcagttc ctcagcctgt cgaccaaggc ccacgacgtg gccttcatcc
      301 a
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LOCUS       UBA545007               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_M_4_A1Y.
ACCESSION   AJ545007
VERSION     AJ545007.1  GI:28556816
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545006               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_L_3_A1Y.
ACCESSION   AJ545006
VERSION     AJ545006.1  GI:28556814
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
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            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545005               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_J_23_A1Y.
ACCESSION   AJ545005
VERSION     AJ545005.1  GI:28556812
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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      121 gtatgtcggc ggtgcgtttc gatcattgcc tgctgtatgg tgatgaacta caagccactt
      181 atgagttgtt taccgaggta ctcggctttt acctggccga gcaagtggtc gatgccgagg
      241 gtatacgcct agcacagttt ctaagcttgt cgaccaaggc ccacgatgtg gcttttatcc
      301 a
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LOCUS       UBA545004               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_G_28_A1Y.
ACCESSION   AJ545004
VERSION     AJ545004.1  GI:28556809
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
//
LOCUS       UBA545003               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ545003
VERSION     AJ545003.1  GI:28556807
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ORIGIN
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       61 atactggaaa gtgggggtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
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      301 a
//
LOCUS       UBA545002                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ545002
VERSION     AJ545002.1  GI:28556804
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
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  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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BASE COUNT       58 a     92 c     93 g     57 t      1 others
ORIGIN
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       61 acaccggcaa gtgggggtg gaagagatca acccggaagc ctggccgcgc aacctcaaag
      121 gcatgcgcgc ggtgcgtttc gaccactgcc tgatgtatgg cgacgagctg cargcgacct
      181 atgatctgtt caccgaggtg ctcggtttct atctggccga gcaggtgatc gacgacaacg
      241 gtacgcgcat cgcgcagttc ctcagcctgt cgaccaaggc ccacgacgtg gccttcatcc
      301 a
//
LOCUS       UBA545001                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_C_5_A1Y.
ACCESSION   AJ545001
VERSION     AJ545001.1  GI:28556802
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
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  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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ORIGIN
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       61 acactggcaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc aatctgaaag
      121 gcatgcccgc ggtgcgtttc gaccattgcc tgctgtatgg cgacgaactg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgacaacg
      241 gcacgcgcgt cgcgcagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA545000                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_G_36_3Y.
ACCESSION   AJ545000
VERSION     AJ545000.1  GI:28556800
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544999                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_F_11_3Y.
ACCESSION   AJ544999
VERSION     AJ544999.1  GI:28556797
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
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            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
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  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544998               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ544998
VERSION     AJ544998.1  GI:28556795
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatgccggc tgtgcgtttc gatcattgcc tgctgtatgg cgacgaactg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgacaacg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
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LOCUS       UBA544997               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ544997
VERSION     AJ544997.1  GI:28556792
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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FEATURES             Location/Qualifiers
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BASE COUNT       57 a     92 c     95 g     57 t
ORIGIN
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       61 acaccggcaa gtgggggtg gaagagatca acccggaagc ctggccacgc aacctcaagg
      121 gcatgcgcgc ggtgcgtttc gaccactgtc tgctgtacgg cgacgagctg caggcgacct
      181 acaatctgtt caccgaggtg ctcggtttct atctggccga gcaggtggtc gacgacaacg
      241 gtacgcgcat tgcgcagttc ctcagcctgt cgaccaaggc ccacgacgtg gccttcatcc
      301 a
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LOCUS       UBA544996               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_F_56_3X.
ACCESSION   AJ544996
VERSION     AJ544996.1  GI:28556790
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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BASE COUNT       64 a     84 c     90 g     63 t
ORIGIN
        1 gacgggtgcg cttccaggca ccctccgggc atcacttcga gttgtatgca gacaaggaat
       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gaccactgcc taatgtatgg cgacgaattg ccggccacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgatg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544995                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ544995
VERSION     AJ544995.1  GI:28556788
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       56 a     93 c     92 g     60 t
ORIGIN
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       61 acaccggcaa gtggggcgtg aatgaggtca atcctgaggc ctggccgcgc gatctcaagg
      121 gcatgcgcgc ggtgcgtttc gaccactgcc tgatgtatgg cgacgagttg ccggccacct
      181 atgacctgtt caccgaggtg ctcggtttct acctggccga acaggtgatc gacgacaacg
      241 gcacgcgcat cgcccagttc ctcagcctgt cgaccaaggc ccatgacgtg gccttcatcc
      301 a
//
LOCUS       UBA544994                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_G_22_3X.
ACCESSION   AJ544994
VERSION     AJ544994.1  GI:28556785
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
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      301 a
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LOCUS       UBA544993                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_B_1_3X.
ACCESSION   AJ544993
VERSION     AJ544993.1  GI:28556783
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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      301 a
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LOCUS       UBA544992                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_M_2_1Y.
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ACCESSION   AJ544992
VERSION     AJ544992.1  GI:28556781
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
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  TITLE     Functional structure in BTEX contaminated soils by means of
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  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
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      301 a
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LOCUS       UBA544991               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
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ACCESSION   AJ544991
VERSION     AJ544991.1  GI:28556778
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544990               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_I_6_1Y.
ACCESSION   AJ544990
VERSION     AJ544990.1  GI:28556776
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>301
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544989               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_H_7_1Y.
ACCESSION   AJ544989
VERSION     AJ544989.1  GI:28556774
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544988                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_G_31_1Y.
ACCESSION   AJ544988
VERSION     AJ544988.1  GI:28556771
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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BASE COUNT       61 a     84 c     91 g     65 t
ORIGIN
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       61 atactggaaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc tactgtatgg cgacgaattg ccggcgacct
      181 atgacctgtt caccaaggtg ctcggcttct atctggccga acaggtgctg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc ccacgacgtg gccttcattc
      301 a
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LOCUS       UBA544987                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_F_11_1Y.
ACCESSION   AJ544987
VERSION     AJ544987.1  GI:28556769
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>301
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BASE COUNT       59 a     90 c     94 g     58 t
ORIGIN
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       61 acaccggcaa gtgggggggtg gaagagatca acccggaagc ctggccacgc aacctcaaag
      121 gcatgcgtgc ggtgcgtttc gaccactgcc tgatgtatgg cgatgagctg caagccacct
      181 atgagctgtt caccgaggtg ctcggtttct acctggccga gcaggtgatc gacgacgacg
      241 gcacccgcgt cgcgcagttc ctcagcctgt cgaccaaagc gcacgacgtg gccttcatcc
      301 a
//
LOCUS       UBA544986                301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_E_11_1Y.
ACCESSION   AJ544986
VERSION     AJ544986.1  GI:28556767
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     gene            1..301
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     CDS             <1..>301
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                     /codon_start=3
                     /transl_table=11
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BASE COUNT       62 a     81 c     91 g     67 t
ORIGIN
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       61 atactggcaa gtggggtgtg aatgaggtca atcccgaggc atggccgcgc gatctgaaag
      121 gcatggcggc tgtgcgtttc gatcattgcc taatgtatgg cgatgaattg ccggccacct
      181 atgacctgtt caccaaggtg ctcggtttct atctggccga acaggtgatg gacgaaaatg
      241 gcacgcgcgt cgcccagttc ctcagtctgt cgaccaaggc gcacgacgtg gccttcattc
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LOCUS       UBA544985               301 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C23O gene for catechol 2,3
            dioxygenase, band SS_D_24_1Y.
ACCESSION   AJ544985
VERSION     AJ544985.1  GI:28556764
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 301)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
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     gene            1..301
                     /gene="C23O"
     CDS             <1..>301
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                     /transl_table=11
                     /product="catechol 2,3 dioxygenase"
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BASE COUNT       57 a     91 c     95 g     58 t
ORIGIN
        1 ggcgggtgcg cttccaggtg ccgtccgggc attttttga gctctacgcc gacaaggaat
       61 acaccggcaa gtgggggtg gaagagatca acccggaagc ctggccacgc aacctcaagg
      121 gcatgcgcgc ggtgcgtttc gaccactgtc tgctgtacgg cgacgagctg caggcgacct
      181 acaatctgtt caccgaggtg ctcggtttct atctggccga gcaggtggtc gacgacaacg
      241 gtacgcgcat tgcgcagttc ctcagcctgt cgaccaaggc ccacgacgtg gccttcatcc
      301 a
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LOCUS       UBA544984               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 37-3Y.
ACCESSION   AJ544984
VERSION     AJ544984.1  GI:28556762
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
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                     /environmental_sample
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     CDS             <1..>515
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                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      105 a    142 c    156 g    112 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggggt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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LOCUS       UBA544983               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 36-3Y.
ACCESSION   AJ544983
VERSION     AJ544983.1  GI:28556760
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT       98 a    155 c    160 g    102 t
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      241 ggcatgcgcg ccgtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
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LOCUS       UBA544982               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 35-3Y.
ACCESSION   AJ544982
VERSION     AJ544982.1  GI:28556757
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
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  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFHLETWED
                     VLRAADLISMT"
BASE COUNT      106 a    144 c    154 g    111 t
ORIGIN
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       61 ctgatggcat atggctgtgc cgttgagcag ctacccgcag gtgaactgaa cagttgtggc
      121 cgacgggtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggtatggcgg ctgtgcgttt cgaccacgcc ctcatgtatg gcgacgaatt gccggcgacc
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      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
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LOCUS       UBA544981               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 35-1Y.
ACCESSION   AJ544981
VERSION     AJ544981.1  GI:28556755
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     MLRAADLISMT"
BASE COUNT      102 a    150 c    158 g    105 t
ORIGIN
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      121 cggcgggtgc gcttccaggt gccgtccggg catttttttg agctctacgc cgacaaggaa
      181 tacaccggca agtgggggat ggaagagatc aacccggaag cctggccacg caacctcaaa
      241 ggcatgcgtg cggtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
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LOCUS       UBA544980               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 34-1Y.
ACCESSION   AJ544980
VERSION     AJ544980.1  GI:28556752
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      101 a    150 c    158 g    106 t
ORIGIN
        1 atggatttta tggctttcaa ggtgctcgac gaggactgtc tgtatcagct gacccaggac
       61 ctgctcgatt atggctgcat gatcgaaagc atccctgccg gtgagctcaa cgggtgcggc
      121 cggcgggtgc gcttccaggt gccgtccggg catttttttg agctctacgc cgacaaggaa
      181 tacaccggca agtgggggat ggaagagatc aacccggaag cctggccacg caacctcaaa
      241 ggcatgcgtg cggtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
      481 gtgctgcgcg cagccgacct gatctccatg accga
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LOCUS       UBA544979               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 33-1Y.
ACCESSION   AJ544979
VERSION     AJ544979.1  GI:28556750
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
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  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     gene            1..515
                     /gene="C230"
     CDS             <1..>515
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEPWPRDLKGMAAVRFDHCLLYGDELPATYD
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                     VLRAADLISMT"
BASE COUNT      104 a    144 c    153 g    114 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagc catggccgcg cgatctgaaa
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      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
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      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
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LOCUS       UBA544978               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 32-3Y.
ACCESSION   AJ544978
VERSION     AJ544978.1  GI:28556748
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      109 a    125 c    155 g    126 t
ORIGIN
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      241 ggtatgtcgg cggtgcgttt cgatcattgc ctgctgtatg gtgatgaact acaagccact
      301 tatgagttgt ttaccgaggt actcggcttt tacctggccg agcaagtggt cgatgccgag
      361 ggtatacgcc tagcacagtt tctaagcttg tcgaccaagg cccacgatgt ggcttttatc
      421 cagcatgcgg agaagggtaa gttccatcat gcctcattcc tcctcgatac ctgggaggac
      481 gtgttgcgcg ctgccgacct gatcagcatg accga
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LOCUS       UBA544977               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 32-1Y.
ACCESSION   AJ544977
VERSION     AJ544977.1  GI:28556745
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      101 a    149 c    160 g    105 t
ORIGIN
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LOCUS       UBA544976               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 31-1Y.
ACCESSION   AJ544976
VERSION     AJ544976.1  GI:28556743
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     VLRAADLISMT"
BASE COUNT      101 a    149 c    159 g    106 t
ORIGIN
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      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
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LOCUS       UBA544975               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544975
VERSION     AJ544975.1  GI:28556741
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      104 a    144 c    154 g    113 t
ORIGIN
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      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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LOCUS       UBA544974               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 28-1Y.
ACCESSION   AJ544974
VERSION     AJ544974.1  GI:28556738
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:77133"
                     /clone="28-1Y"
                     /environmental_sample
     gene            1..515
                     /gene="C230"
     CDS             <1..>515
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                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    142 c    156 g    113 t
ORIGIN
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      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
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LOCUS       UBA544973               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 27-3Y.
ACCESSION   AJ544973
VERSION     AJ544973.1  GI:28556736
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
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DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544972
VERSION     AJ544972.1  GI:28556734
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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LOCUS       UBA544971               515 bp    DNA     linear   BCT 03-JUL-2003
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ACCESSION   AJ544971
VERSION     AJ544971.1  GI:28556731
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 26-1Y.
ACCESSION   AJ544970
VERSION     AJ544970.1  GI:28556729
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544969
VERSION     AJ544969.1  GI:28556727
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ACCESSION   AJ544968
VERSION     AJ544968.1  GI:28556724
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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VERSION     AJ544967.1  GI:28556722
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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LOCUS       UBA544966               515 bp    DNA     linear   BCT 03-JUL-2003
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ACCESSION   AJ544966
VERSION     AJ544966.1  GI:28556719
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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LOCUS       UBA544965               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544965
VERSION     AJ544965.1  GI:28556717
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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                     /db_xref="GI:28556718"
                     /translation="MDFMGFKVLDEDCLYQLTQDLLDYGCMIESIPAGELNGCGRRVR
                     FQVPSGHFFELYADKEYTGKWGVEEINPEAWPRNLKGMRAVRFDHCLMYGDELQATYE
                     LFTEVLGFYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWED
                     VLRAADLISMT"
BASE COUNT      101 a    149 c    160 g    105 t
ORIGIN
        1 atggatttta tggggttcaa ggtgctcgac gaggactgtc tgtatcagct gacccaggac
       61 ctgctcgatt atggctgcat gatcgaaagc atccctgccg gtgagctcaa cgggtgcggc
      121 cggcgggtgc gcttccaggt gccgtccggg catttttttg agctctacgc cgacaaggaa
      181 tacaccggca agtggggggt ggaagagatc aacccggaag cctggccacg caacctcaaa
      241 ggcatgcgtg cggtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
      481 gtgctgcgcg cagccgacct gatctccatg accga
//
LOCUS       UBA544964               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 21-1Y.
ACCESSION   AJ544964
VERSION     AJ544964.1  GI:28556715
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
     source          1..515
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                     /db_xref="taxon:77133"
                     /clone="21-1Y"
                     /environmental_sample
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                     /gene="C230"
     CDS             <1..>515
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                     /codon_start=1
                     /transl_table=11
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                     /db_xref="GI:28556716"
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      103 a    142 c    157 g    113 t
ORIGIN
        1 atggatttta tggggttcaa ggttgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaggac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
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LOCUS       UBA544963               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 20-3Y.
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ACCESSION   AJ544963
VERSION     AJ544963.1  GI:28556712
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
     source          1..515
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:77133"
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                     /environmental_sample
     gene            1..515
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     CDS             <1..>515
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                     /transl_table=11
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                     /protein_id="CAD67876.1"
                     /db_xref="GI:28556713"
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    143 c    155 g    113 t
ORIGIN
        1 atggatttta tgggcttcaa ggttgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544962               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 20-1Y.
ACCESSION   AJ544962
VERSION     AJ544962.1  GI:28556709
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
     source          1..515
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                     /db_xref="taxon:77133"
                     /clone="20-1Y"
                     /environmental_sample
     gene            1..515
                     /gene="C230"
     CDS             <1..>515
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                     /codon_start=1
                     /transl_table=11
                     /product="catechol 2,3 dioxygenase"
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                     FQAPSGHFFELYADKEYTGKWGLAEVNPEAWPRNLKGMRAVRFDHCLMYGDELQATYE
                     LFTEVLGFYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWED
                     VLRAADLISMT"
BASE COUNT       93 a    164 c    160 g     98 t
ORIGIN
        1 atggatttta tggcgttcaa ggtgctcgac gacgccaccc tgagcagcct caccgacgac
       61 ctgatccgct acggctgcct ggtcgagctg atcgccgccg gcgagctcaa ggactgcggt
      121 cggcgggtgc gcttccaggc gccttccggg cacttcttcg agctctatgc ggacaaggag
      181 tacaccggca aatggggctt ggccgaggtc aacccggagg cctggccgcg caacctcaaa
      241 ggcatgcgtg cggtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
      481 gtgctgcgcg cagccgacct gatctccatg accga
//
LOCUS       UBA544961               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 19-3Y.
ACCESSION   AJ544961
VERSION     AJ544961.1  GI:28556707
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
                     /clone="19-3Y"
                     /environmental_sample
     gene            1..515
                     /gene="C230"
     CDS             <1..>515
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                     /EC_number="1.13.11.2"
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                     /transl_table=11
                     /product="catechol 2,3 dioxygenase"
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                     /translation="MDFMGFKVVDEDALRQLERDLTAYGCAVEQLPAGELNSCGRRVR
                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    143 c    155 g    113 t
ORIGIN
        1 atggatttta tgggcttcaa ggttgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544960               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 19-1Y.
ACCESSION   AJ544960
VERSION     AJ544960.1  GI:28556704
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
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  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:77133"
                     /clone="19-1Y"
                     /environmental_sample
     gene            1..515
                     /gene="C230"
     CDS             <1..>515
                     /gene="C230"
                     /EC_number="1.13.11.2"
                     /codon_start=1
                     /transl_table=11
                     /product="catechol 2,3 dioxygenase"
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                     /db_xref="GI:28556705"
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                     FQAPSGHHFELYVDKKYTGKWGVNEINPEAWPRDLKGMAAVRFDHCLLYGDELQATND
                     LFTEVLGFYLAEQVIDAEGKRLAQFLSLSTKAHDVAFIQHAEKGKLHHASFLLESWED
                     VLRAADLISMT"
BASE COUNT      117 a    120 c    151 g    127 t
ORIGIN
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       61 ctgctggcat atggctgtgc catcgagcat gtaccggcgg gcgaactgaa cggctgcggt
      121 aggcgcgtgc gcttccaggc cccctctgga catcacttcg agttgtatgt ggacaagaaa
      181 tacactggta aatggggtgt gaatgagatt aatcccgagg cctggccgcg cgatctgaaa
      241 gggatggcgg ctgtacgctt cgaccattgc ttgctgtatg gcgacgaatt acaagccaca
      301 aatgatctgt ttaccgaggt gttaggcttc tatctagccg agcaagtaat cgatgcagaa
      361 ggtaaacgcc tagctcaatt tctcagtttg tcgaccaagg cacatgatgt tgcttttatt
      421 cagcatgcgg agaagggcaa gctccatcat gcctcattcc tccttgagag ttgggaggat
      481 gtgttgcgcg ccgccgacct gatcagcatg accga
//
LOCUS       UBA544959               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 17-1Y.
ACCESSION   AJ544959
VERSION     AJ544959.1  GI:28556702
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="17-1Y"
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     CDS             <1..>515
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                     /transl_table=11
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      105 a    142 c    156 g    112 t
ORIGIN
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       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgaactgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggggt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544958               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 16-1Y.
ACCESSION   AJ544958
VERSION     AJ544958.1  GI:28556699
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="16-1Y"
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     gene            1..515
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     CDS             <1..>515
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                     LFTEVLGFYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWED
                     VLRAADLISMT"
BASE COUNT       93 a    163 c    160 g     99 t
ORIGIN
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      241 ggcatgcgtg cggtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
      481 gtgctgcgcg cagccgacct gatctccatg accga
//
LOCUS       UBA544957               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 14-3Y.
ACCESSION   AJ544957
VERSION     AJ544957.1  GI:28556697
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
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  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     gene            1..515
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     CDS             <1..>515
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                     FRAPSGHFFELYADKQYTGKWGVEEINPEAWPRDLKGMRAVRFDHCLMYGDELQATYE
                     LFTEVLGFYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWED
                     VLRAADLISMT"
BASE COUNT       98 a    155 c    160 g    102 t
ORIGIN
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       61 ctgctcaact atggctgtct ggtcgagagt atcgccgccg gcgaactcaa ggggtgtggc
      121 cgacgggtgc gcttccgggc accgtccggg cacttcttcg agctctatgc ggacaagcaa
      181 tacaccggta aatggggggt cgaggagatc aaccccgagg cctggccgcg cgatttaaag
      241 ggcatgcgcg ccgtgcgttt cgaccactgc ctgatgtatg gcgatgagct gcaagccacc
      301 tatgagctgt tcaccgaggt gctcggtttc tacctggccg agcaggtgat cgacgacgac
      361 ggcacccgcg tcgcgcagtt cctcagcctg tcgaccaaag cgcacgacgt ggccttcatc
      421 cattgcccgg agaagggcaa gttccaccat gtgtcgttct tcctggaaac ctgggaggac
      481 gtgctgcgcg cagccgacct gatctccatg accga
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LOCUS       UBA544956               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 14-1Y.
ACCESSION   AJ544956
VERSION     AJ544956.1  GI:28556695
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:77133"
                     /clone="14-1Y"
                     /environmental_sample
     gene            1..515
                     /gene="C230"
     CDS             <1..>515
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                     /db_xref="GI:28556696"
                     /translation="MDFMAFKVVDEDALRQLERDLTAYGCAVEQLPAGELNSCGRRVR
                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      103 a    143 c    156 g    113 t
ORIGIN
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       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaggac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544955               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 13-1Y.
ACCESSION   AJ544955
VERSION     AJ544955.1  GI:28556692
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /environmental_sample
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     CDS             <1..>515
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                     /EC_number="1.13.11.2"
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                     /protein_id="CAD67868.1"
                     /db_xref="GI:28556693"
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                     VLRAADLISMT"
BASE COUNT      105 a    143 c    153 g    114 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544954               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 12-3Y.
ACCESSION   AJ544954
VERSION     AJ544954.1  GI:28556690
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
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                     /environmental_sample
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     CDS             <1..>515
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                     /transl_table=11
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                     /protein_id="CAD67867.1"
                     /db_xref="GI:28556691"
                     /translation="MDFMGFKVLDEDCLYQLTQDLLDYGCMIESIPAGELNGCGRRVR
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                     VLRAADLISMT"
BASE COUNT      101 a    154 c    154 g    106 t
ORIGIN
        1 atggatttta tggggttcaa ggtgctcgac gaggactgtc tgtatcagct gacccaggac
       61 ctgctcgatt atggctgcat gatcgaaagc atccctgccg gtgagctcaa cgggtgcggc
      121 cggcgggtgc gcttccaggt gccgtccggg catttttttg agctctacgc tgacaaggaa
      181 tacaccggca agtggggggt ggaagagacc aacccggaag cctggccacg caacctcaag
      241 ggcatgcgcg cggtgcgttt cgaccactgt ctgctgtacg gcgacgagct gcaggcgacc
      301 tacaatctgt tcaccgaggt gctcggtttc tatctggccg agcaggtggt cgacgacaac
      361 ggtacgcgca ttgcgcagtt cctcagcctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544953               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 12-1Y.
ACCESSION   AJ544953
VERSION     AJ544953.1  GI:28556688
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /db_xref="taxon:77133"
                     /clone="12-1Y"
                     /environmental_sample
     gene            1..515
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     CDS             <1..>515
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    142 c    155 g    114 t
ORIGIN
        1 atggatttta tgggtttcaa ggttgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544952               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 11-1Y.
ACCESSION   AJ544952
VERSION     AJ544952.1  GI:28556685
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
                     /clone="11-1Y"
                     /environmental_sample
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                     /gene="C230"
     CDS             <1..>515
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                     /EC_number="1.13.11.2"
                     /codon_start=1
                     /transl_table=11
                     /product="catechol 2,3 dioxygenase"
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                     /db_xref="GI:28556686"
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                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    144 c    154 g    113 t
ORIGIN
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       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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LOCUS       UBA544951               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 10-3Y.
ACCESSION   AJ544951
VERSION     AJ544951.1  GI:28556683
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="10-3Y"
                     /environmental_sample
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      104 a    142 c    155 g    114 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
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      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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//
LOCUS       UBA544950               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 09-3Y.
ACCESSION   AJ544950
VERSION     AJ544950.1  GI:28556681
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="09-1Y"
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      109 a    125 c    155 g    126 t
ORIGIN
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      121 cggcgtgtgc gcttccaagc cccctcaggg catcacttcg agttgtatgc tgacaaggaa
      181 tacactggca aatggggtgt gaatgaggtc aatcccgaag cctggccacg cgatctgaaa
      241 ggtatgtcgg cggtgcgttt cgatcattgc ctgctgtatg gtgatgaact acaagccact
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//
LOCUS       UBA544949               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 09-1Y.
ACCESSION   AJ544949
VERSION     AJ544949.1  GI:28556678
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="09-1Y"
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     gene            1..515
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     CDS             <1..>515
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                     /db_xref="GI:28556679"
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                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    142 c    156 g    113 t
ORIGIN
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       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544948               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 08-3Y.
ACCESSION   AJ544948
VERSION     AJ544948.1  GI:28556676
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /clone="08-3Y"
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     CDS             <1..>515
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                     /protein_id="CAD67861.1"
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
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LOCUS       UBA544947               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 08-1Y.
ACCESSION   AJ544947
VERSION     AJ544947.1  GI:28556674
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     VLRAADLISMT"
BASE COUNT      105 a    143 c    155 g    112 t
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       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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LOCUS       UBA544946               512 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 07-3Y.
ACCESSION   AJ544946
VERSION     AJ544946.1  GI:28556671
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 512)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>512
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                     LRAADLISMT"
BASE COUNT       85 a    167 c    165 g     95 t
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      181 gtcaccggca agtggggcct cgaagaggtc aacccgggag cctggccgcg cggcctcaag
      241 ggcatgcgtg cgcagcgctt cgaccattgc ctgctgtacg gcgacgaact cgacgagacc
      301 ctgcgcctgt tcaccgaggt gctcggcttc accctcgccg aacaggtgct cgatggcggc
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      421 cacccggaga agggccgttt ccaccatgcc tcgttcttcc tcgagacctg ggaagacgtg
      481 ctgcgcgccg ccgacctgat cagcatgacc ga
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LOCUS       UBA544945               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 07-1Y.
ACCESSION   AJ544945
VERSION     AJ544945.1  GI:28556669
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     VLRAADLISMT"
BASE COUNT      114 a    122 c    155 g    124 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggga
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagc catggccgcg cgatctgaaa
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      421 cagcacgcgg agaagggcaa gctccatcat gcctcattcc tccttgagag ttgggaggat
      481 gtgttgcgcg ccgccgacct gatcagcatg accga
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LOCUS       UBA544944               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544944
VERSION     AJ544944.1  GI:28556666
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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                     VLRAADLISMT"
BASE COUNT      104 a    141 c    155 g    115 t
ORIGIN
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
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      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
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LOCUS       UBA544943               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 06-1Y.
ACCESSION   AJ544943
VERSION     AJ544943.1  GI:28556664
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
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                     VLRAADLISMT"
BASE COUNT      104 a    143 c    156 g    112 t
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      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtgggggct gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
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DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
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ACCESSION   AJ544942
VERSION     AJ544942.1  GI:28556662
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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     CDS             <1..>515
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                     LFTEVLGFYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWED
                     VLRAADLISMT"
BASE COUNT       93 a    164 c    160 g     98 t
ORIGIN
        1 atggatttta tggcgttcaa ggtgctcgac gacgccaccc tgagcagcct caccgacgac
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LOCUS       UBA544941               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 02-3Y.
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ACCESSION   AJ544941
VERSION     AJ544941.1  GI:28556659
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     VLRAADLISMT"
BASE COUNT      109 a    125 c    155 g    126 t
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      181 tacactggca aatggggtgt gaatgaggtc aatcccgaag cctggccacg cgatctgaaa
      241 ggtatgtcgg cggtgcgttt cgatcattgc ctgctgtatg gtgatgaact acaagccact
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LOCUS       UBA544940               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 02-1Y.
ACCESSION   AJ544940
VERSION     AJ544940.1  GI:28556657
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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                     /clone="02-1Y"
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                     /protein_id="CAD67853.1"
                     /db_xref="GI:28556658"
                     /translation="MDFMAFKVVDEDALRQLERDLTAYGCAVEQLPAGELNSCGRRVR
                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    145 c    154 g    112 t
ORIGIN
        1 atggatttta tggccttcaa ggtcgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544939               515 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Uncultured bacterium partial C230 gene for catechol 2,3
            dioxygenase, clone 01-1Y.
ACCESSION   AJ544939
VERSION     AJ544939.1  GI:28556654
KEYWORDS    C230 gene; catechol 2,3 dioxygenase.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 515)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:77133"
                     /clone="01-1Y"
                     /environmental_sample
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     CDS             <1..>515
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                     /db_xref="GI:28556655"
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                     FQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYD
                     LFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWED
                     VLRAADLISMT"
BASE COUNT      104 a    142 c    156 g    113 t
ORIGIN
        1 atggatttta tggggttcaa ggttgtggat gaggatgctc tccggcaact ggagcgggat
       61 ctgacggcat atggctgtgc cgttgagcag ctacccgcag gtgagctgaa cagttgtggc
      121 cggcgcgtgc gcttccaggc accctccggg catcacttcg agttgtatgc agacaaggaa
      181 tatactggaa agtggggtgt gaatgaggtc aatcccgagg catggccgcg cgatctgaaa
      241 ggcatggcgg ctgtgcgttt cgatcattgc ctactgtatg gcgacgaatt gccggcgacc
      301 tatgacctgt tcaccaaggt gctcggcttc tatctggccg aacaggtgct ggacgaaaat
      361 ggcacgcgcg tcgcccagtt cctcagtctg tcgaccaagg cccacgacgt ggccttcatt
      421 caccatccgg aaaaaggccg cctccatcat gtgtccttct acctcgaaac ctgggaagac
      481 gtgcttcgcg ccgccgacct gatctccatg accga
//
LOCUS       UBA544938               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. A1YC1 partial C23O gene for catechol 2,3
            dioxygenase, strain A1YC1.
ACCESSION   AJ544938
VERSION     AJ544938.1  GI:28556652
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. A1YC1
  ORGANISM  Pseudomonas sp. A1YC1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
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  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:237012"
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     CDS             <1..>818
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                     /protein_id="CAD67851.1"
                     /db_xref="GI:28556653"
                     /translation="RVLNLESALAHYRDLLGLIEVDRDDFGRVYLKAWTEVDKFSLVL
                     READEPGMDFMGFKVVDEDALRQLERDLMAYGCAVEQLPAGELNSCGRRVRFQAPSGH
                     HFELYSDKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHALMYGDELPATYDLFTKVLG
                     FYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFHLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDPSGNRNEVFCGGDYNYPDHKPVTWTTD"
BASE COUNT      171 a    243 c    236 g    168 t
ORIGIN
        1 cgcgtactca atctagagag tgccctggcc cactaccgcg acctgctcgg cctgatcgaa
       61 gtggaccgtg atgacttcgg gcgcgtctat ttgaaggctt ggaccgaagt ggataagttt
      121 tccctggtgc tgcgtgaagc ggatgagccg ggcatggatt tcatgggttt caaggttgtg
      181 gatgaggatg ctctccggca actggagcgg gatctgatgg catatggctg tgccgttgag
      241 cagctacccg caggtgaact gaacagttgt ggccgacggg tgcgcttcca ggcaccctcc
      301 gggcatcact tcgagttgta ttcagacaag gaatatactg gaaagtgggg tgtgaatgag
      361 gtcaatcccg aggcatggcc gcgcgatctg aaaggtatgg cggctgtgcg tttcgaccac
      421 gccctcatgt atggcgacga attgccggcg acctatgacc tgttcaccaa ggtgctcggc
      481 ttctatctgg ccgaacaggt gctggacgaa aatggcacgc gcgtcgccca gttcctcagt
      541 ctgtcgacca aggcccacga cgtggccttc attcaccatc cggaaaaagg ccgcctccat
      601 catgtgtcct tccacctcga aacctgggaa gacgtgcttc gcgccgccga cctgatctcc
      661 atgaccgaca cctcgatcga catcggccca acccgccacg gcctcactca cggcaagacc
      721 atctacttct tcgacccgtc cggtaaccgc aacgaagtgt tctgcggggg agattacaac
      781 tacccggacc acaaaccggt gacctggacc accgacca
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LOCUS       UBA544937               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 3YXyl3 partial C23O gene for catechol 2,3
            dioxygenase, strain 3YXyl3.
ACCESSION   AJ544937
VERSION     AJ544937.1  GI:28556650
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 3YXyl3
  ORGANISM  Pseudomonas sp. 3YXyl3
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /transl_table=11
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                     /protein_id="CAD67850.1"
                     /db_xref="GI:28556651"
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                     READEPGMDFMGFKVVDEDALRQLERDLTAYGCAVEQLPAGELNSCGRRVRFQAPSGH
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                     FYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDPSGNRSEVFCGGNYSYPDHKPVTWLAK"
BASE COUNT      169 a    234 c    242 g    173 t
ORIGIN
        1 cgtgtactgg acatgagcaa ggccctggaa cactacgtcg agttgctggg cctgatcgag
       61 atggaccgtg acgatcaggg ccgtgtctat ctcaaggctt ggaccgaagt tgacaaattc
      121 tccgtggtgc tacgcgaagc cgacgaacct ggtatggatt ttatgggttt caaggttgtg
      181 gatgaggatg ctctccggca actggagcgg gatctgacgg catatggctg tgccgttgag
      241 cagctacccg caggtgagct gaacagttgt ggccggcgcg tgcgcttcca ggcaccctcc
      301 gggcatcact tcgagttgta tgcagacaag gaatatactg gaaagtgggg tgtgaatgag
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      421 tgcctactgt atggcgacga attgccggcg acctatgacc tgttcaccaa ggtgctcggc
      481 ttctatctgg ccgaacaggt gctggacgaa aatggcacgc gcgtcgccca gttcctcagt
      541 ctgtcgacca aggcccacga cgtggccttc attcaccatc cggaaaaagg ccgcctccat
      601 catgtgtcct tctacctcga aacctgggaa gacgtgcttc gcgccgccga cctgatctcc
      661 atgaccgaca cctctatcga catcggcccg acccgtcatg gcctgaccca cggcaagacc
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LOCUS       UBA544936               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 3YXyl2 partial C23O gene for catechol 2,3
            dioxygenase, strain 3YXyl2.
ACCESSION   AJ544936
VERSION     AJ544936.1  GI:28556648
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 3YXyl2
  ORGANISM  Pseudomonas sp. 3YXyl2
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     FYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDPSGNRSEVFCGGNYSYPDHKPVTWLAK"
BASE COUNT      169 a    234 c    242 g    173 t
ORIGIN
        1 cgtgtactgg acatgagcaa ggccctggaa cactacgtcg agttgctggg cctgatcgag
       61 atggaccgtg acgatcaggg ccgtgtctat ctcaaggctt ggaccgaagt tgacaaattc
      121 tccgtggtgc tacgcgaagc cgacgaacct ggtatggatt ttatgggttt caaggttgtg
      181 gatgaggatg ctctccggca actggagcgg gatctgacgg catatggctg tgccgttgag
      241 cagctacccg caggtgagct gaacagttgt ggccggcgcg tgcgcttcca ggcaccctcc
      301 gggcatcact tcgagttgta tgcagacaag gaatatactg gaaagtgggg tgtgaatgag
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      661 atgaccgaca cctctatcga catcggcccg acccgtcatg gcctgaccca cggcaagacc
      721 atctacttct tcgacccgtc cggtaaccgc agcgaggtgt tctgcggcgg caactacagc
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LOCUS       UBA544935               818 bp    DNA     linear   BCT 03-JUL-2003
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DEFINITION  Pseudomonas sp. 3YXyl1 partial C23O gene for catechol 2,3
            dioxygenase, strain 3YXyl1.
ACCESSION   AJ544935
VERSION     AJ544935.1  GI:28556645
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 3YXyl1
  ORGANISM  Pseudomonas sp. 3YXyl1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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                     HFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHCLLYGDELPATYDLFTKVLG
                     FYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDPSGNRSEVFCGGNYSYPDHKPVTWLAK"
BASE COUNT      169 a    234 c    242 g    173 t
ORIGIN
        1 cgtgtactgg acatgagcaa ggccctggaa cactacgtcg agttgctggg cctgatcgag
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      601 catgtgtcct tctacctcga aacctgggaa gacgtgcttc gcgccgccga cctgatctcc
      661 atgaccgaca cctctatcga catcggcccg acccgtcatg gcctgaccca cggcaagacc
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LOCUS       UBA544934               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 3YdBTEX2 partial C23O gene for catechol 2,3
            dioxygenase, strain 3YdBTEX2.
ACCESSION   AJ544934
VERSION     AJ544934.1  GI:28556643
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 3YdBTEX2
  ORGANISM  Pseudomonas sp. 3YdBTEX2
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
FEATURES             Location/Qualifiers
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                     /protein_id="CAD67847.1"
                     /db_xref="GI:28556644"
                     /translation="RVLNLESALTHYRDLLGLIETDRDEQGRIYLKAWSEVDKFSVVL
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                     FFELYADKEYTGKWGLAEVNPEAWPRNLKGMRAVRFDHCLMYGDELQATYELFTEVLG
                     FYLAEQVIDDDGTRVAQFLSLSTKAHDVAFIHCPEKGKFHHVSFFLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDSFGNRNEVFCGGDYNYQDHKPVTWLAK"
BASE COUNT      156 a    264 c    248 g    150 t
ORIGIN
        1 cgcgtgttga atctggagtc tgcgctaacg cattaccgcg acctgctcgg cctgatcgag
       61 acggatcgcg acgagcaggg acgtatctac ctgaaggcct ggagcgaggt ggacaagttc
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      241 ctgatcgccg ccggcgagct caaggactgc ggtcggcggg tgcgcttcca ggcgccttcc
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      541 ctgtcgacca aagcgcacga cgtggccttc atccattgcc cggagaaggg caagttccac
      601 catgtgtcgt tcttcctgga aacctgggag gacgtgctgc gcgcagccga cctgatctcc
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      721 atctacttct tcgactcctt cggcaaccgc aacgaggtgt tctgtggcgg cgattacaac
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LOCUS       UBA544933               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 3YdBTEX1b partial C23O gene for catechol 2,3
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ACCESSION   AJ544933
VERSION     AJ544933.1  GI:28556426
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 3YdBTEX1b
  ORGANISM  Pseudomonas sp. 3YdBTEX1b
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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ACCESSION   AJ544932
VERSION     AJ544932.1  GI:28556424
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  ORGANISM  Pseudomonas sp. 1YXyl1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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  ORGANISM  Pseudomonas sp. 1YdBTEX3
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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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BASE COUNT      169 a    234 c    242 g    173 t
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VERSION     AJ544930.1  GI:28556419
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  ORGANISM  Pseudomonas sp. 1YdBTEX2
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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  ORGANISM  Pseudomonas sp. 1YC2
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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
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  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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  ORGANISM  Pseudomonas sp. 1YBTEX3
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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      541 ctgtcgacca aggcccacga cgtggccttc attcaccatc cggaaaaagg ccgcctccat
      601 catgtgtcct tctacctcga aacctgggaa gacgtgcttc gcgccgccga cctgatctcc
      661 atgaccgaca cctctatcga catcggcccg acccgtcatg gcctgaccca cggcaagacc
      721 atctacttct tcgacccgtc cggtaaccgc agcgaggtgt tctgcggcgg caactacagc
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LOCUS       UBA544926               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 1YBTEX1 partial C23O gene for catechol 2,3
            dioxygenase, strain 1YBTEX1.
ACCESSION   AJ544926
VERSION     AJ544926.1  GI:28556410
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1YBTEX1
  ORGANISM  Pseudomonas sp. 1YBTEX1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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        1 cgtgtactgg acatgagcaa ggccctggaa cactacgtcg agttgctggg cctgatcgag
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LOCUS       UBA544925               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 1YB3 partial C23O gene for catechol 2,3
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ACCESSION   AJ544925
VERSION     AJ544925.1  GI:28556407
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1YB3
  ORGANISM  Pseudomonas sp. 1YB3
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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     CDS             <1..>818
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BASE COUNT      169 a    234 c    242 g    173 t
ORIGIN
        1 cgtgtactgg acatgagcaa ggccctggaa cactacgtcg agttgctggg cctgatcgag
       61 atggaccgtg acgatcaggg ccgtgtctat ctcaaggctt ggaccgaagt tgacaaattc
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LOCUS       UBA544924               818 bp    DNA     linear   BCT 03-JUL-2003
DEFINITION  Pseudomonas sp. 1YB2 partial C23O gene for catechol 2,3
            dioxygenase, strain 1YB2.
ACCESSION   AJ544924
VERSION     AJ544924.1  GI:28556405
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1YB2
  ORGANISM  Pseudomonas sp. 1YB2
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
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                     FYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFYLETWEDVLRAADL
                     ISMTDTSIDIGPTRHGLTHGKTIYFFDPSGNRSEVFCGGNYSYPDHKPVTWLAK"
BASE COUNT      169 a    234 c    242 g    173 t
ORIGIN
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DEFINITION  Pseudomonas sp. 1XdBTEX1 partial C23O gene for catechol 2,3
            dioxygenase, strain 1XdBTEX1.
ACCESSION   AJ544923
VERSION     AJ544923.1  GI:28556402
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1XdBTEX1
  ORGANISM  Pseudomonas sp. 1XdBTEX1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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BASE COUNT      176 a    204 c    246 g    192 t
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DEFINITION  Pseudomonas sp. 1XBTEX1 partial C23O gene for catechol 2,3
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ACCESSION   AJ544922
VERSION     AJ544922.1  GI:28556400
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1XBTEX1
  ORGANISM  Pseudomonas sp. 1XBTEX1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
            PCR-SSCP fingerprints: comparative diversity assessment against
            bacterial isolates and PCR-DNA clone libraries targeting a
            catabolic gene family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
            Biotechnology, Environmental Microbiology, Biodegradation Research
            Group Mascheroder Weg 1, Braunschweig, Lower Saxony, D-38124,
            GERMANY
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BASE COUNT      176 a    204 c    246 g    192 t
ORIGIN
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ACCESSION   AJ544921
VERSION     AJ544921.1  GI:28556398
KEYWORDS    C23O gene; catechol 2,3 dioxygenase.
SOURCE      Pseudomonas sp. 1XB2b
  ORGANISM  Pseudomonas sp. 1XB2b
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Functional structure in BTEX contaminated soils by means of
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            PCR-SSCP fingerprints: comparative diversity assessment against
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 818)
  AUTHORS   Junca,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-FEB-2003) Junca H., GBF German Research Centre for
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BASE COUNT      176 a    204 c    246 g    192 t
ORIGIN
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LOCUS       AY228547               1498 bp    DNA     linear   BCT 18-FEB-2003
DEFINITION  Pseudomonas sp. A2YC1 catechol 2,3-dioxygenase (c23o) gene,
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            (hmsD) gene, partial cds.
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VERSION     AY228547.1  GI:28412117
KEYWORDS    .
SOURCE      Pseudomonas sp. A2YC1
  ORGANISM  Pseudomonas sp. A2YC1
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1498)
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Catechol 2,3 Dioxygenase Gene from the Benzene Degrader Isolate
            A2YC1
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1498)
  AUTHORS   Junca,H. and Pieper,D.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-FEB-2003) Biodegradation Research Group,
            Environmental Microbiology, GBF, Mascheroder Weg 1, Braunschweig,
            Lower Saxony 38124, Germany
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Characterization of the Genetic Potential, Catabolic Structure and Degradative Activities against BTEX in Microbial Communities from Aquifers under Adaptation to Organic Contaminants

Howard Díaz Junca

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